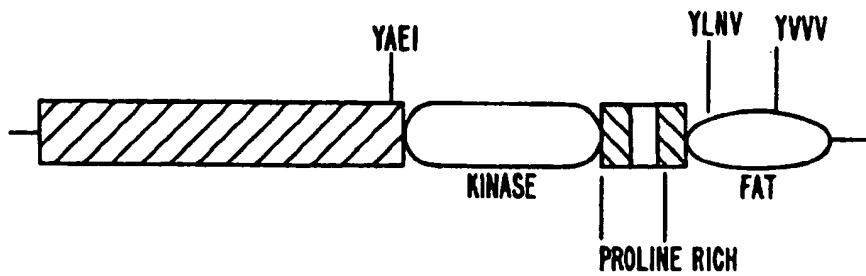


PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/54, 9/12, C12Q 1/68, C07K 16/40, C12N 5/12, G01N 33/68, C12Q 1/48, C07D 41/40, C07C 255/34, C07D 215/00, 239/72	A2	(11) International Publication Number: WO 96/18738 (43) International Publication Date: 20 June 1996 (20.06.96)
(21) International Application Number: PCT/US95/15846 (22) International Filing Date: 6 December 1995 (06.12.95) (30) Priority Data: 08/357,642 15 December 1994 (15.12.94) US 08/460,626 2 June 1995 (02.06.95) US (71) Applicants: SUGEN, INC. [US/US]; 515 Galveston Drive, Redwood City, CA 94063 (US). NEW YORK UNIVERSITY [US/US]; 550 First Avenue, New York, NY 10016 (US). (72) Inventors: LEV, Sima; 8 Locksley Avenue #1C, San Francisco, CA 94122 (US). SCHLESSINGER, Joseph; 37 Washington Square, New York, NY 10011 (US). (74) Agents: WARBURG, Richard, J. et al.; Lyon & Lyon, First Interstate World Center, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: PROBIN TYROSINE KINASE (PYK2) ITS cDNA CLONING AND ITS USES <div style="text-align: center;">PYK2 </div>		
(57) Abstract <p>The present invention features a method for treatment of an organism having a disease or condition characterized by an abnormality in a signal transduction pathway, wherein the signal transduction pathway includes a PYK2 protein. The invention also features methods for diagnosing such diseases and for screening for agents that will be useful in treating such diseases. The invention also features purified and/or isolated nucleic acid encoding a PYK2 protein.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LJ	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

DESCRIPTION**PROBIN TYROSINE KINASE (PYK2) ITS cDNA CLONING AND ITS USES**Related Application

The present application is a continuation-in-part application of U.S. application Serial No. 08/357,642, filed December 15, 1994, incorporated herein by reference
5 in its entirety, including any drawings.

Field of the Invention

The present invention relates generally to the fields of biology, biochemistry and medicine and more specifically to the field of cellular signal transduction.

10 Background of the Invention

None of the following discussion of the background of the invention is admitted to be prior art to the invention.

Cellular signal transduction is a fundamental
15 mechanism whereby external stimuli that regulate diverse cellular processes are relayed to the interior of cells. One of the key biochemical mechanisms of signal transduction involves the reversible phosphorylation of tyrosine residues on proteins. The phosphorylation state of a
20 protein is modified through the reciprocal actions of tyrosine phosphatases (TPs) and tyrosine kinases (TKs), including receptor tyrosine kinases and non-receptor tyrosine kinases.

Receptor tyrosine kinases (RTKs) belong to a
25 family of transmembrane proteins and have been implicated in cellular signaling pathways. The predominant biological activity of some RTKs is the stimulation of cell growth and proliferation, while other RTKs are involved in arresting growth and promoting differentia-
30 tion. In some instances, a single tyrosine kinase can

inhibit, or stimulate, cell proliferation depending on the cellular environment in which it is expressed.

RTKs are composed of at least three domains: an extracellular ligand binding domain, a transmembrane domain and a cytoplasmic catalytic domain that can phosphorylate tyrosine residues. Ligand binding to membrane-bound receptors induces the formation of receptor dimers and allosteric changes that activate the intracellular kinase domains and result in the self-phosphorylation (autophosphorylation and/or transphosphorylation) of the receptor on tyrosine residues. Individual phosphotyrosine residues of the cytoplasmic domains of receptors may serve as specific binding sites that interact with a host of cytoplasmic signaling molecules, thereby activating various signal transduction pathways.

The intracellular, cytoplasmic, non-receptor protein tyrosine kinases do not contain a hydrophobic transmembrane domain or an extracellular domain and share non-catalytic domains in addition to sharing their catalytic kinase domains. Such non-catalytic domains include the SH2 domains and SH3 domains. The non-catalytic domains are thought to be important in the regulation of protein-protein interactions during signal transduction.

Focal adhesion kinase (FAK) is a cytoplasmic protein tyrosine kinase that is localized to focal adhesions. Schaller, et al., Proc. Natl. Acad. Sci. U.S.A., 89:5192-5196 (1992), incorporated herein by reference in its entirety, including any drawings; Cobb et al., Molecular and Cellular Biology, 14(1):147-155 (1994). In some cells the C-terminal domain of FAK is expressed autonomously as a 41 kDa protein called FRNK and the 140 C-terminal residues of FAK contain a focal adhesion targeting (FAT) domain. The cDNA's encoding FRNK are given in Schaller et al., Molecular and Cellular Biology, 13(2):785-791 (1993), incorporated herein by reference in its entirety, including any drawings. The FAT domain was

identified and said to be required for localization of FAK to cellular focal adhesions in Hilderbrand et al., The Journal of Cell Biology, 123(4):993-1005 (1993).

A central feature of signal transduction is the reversible phosphorylation of certain proteins. Receptor phosphorylation stimulates a physical association of the activated receptor with target molecules, which either are or are not phosphorylated. Some of the target molecules such as phospholipase C γ are in turn phosphorylated and activated. Such phosphorylation transmits a signal to the cytoplasm. Other target molecules are not phosphorylated, but assist in signal transmission by acting as adapter molecules for secondary signal transducer proteins. For example, receptor phosphorylation and the subsequent allosteric changes in the receptor recruit the Grb-2/SOS complex to the catalytic domain of the receptor where its proximity to the membrane allows it to activate ras. The secondary signal transducer molecules generated by activated receptors result in a signal cascade that regulates cell functions such as cell division or differentiation. Reviews describing intracellular signal transduction include Aaronson, Science, 254:1146-1153, 1991; Schlessinger, Trends Biochem. Sci., 13:443-447, 1988; and Ullrich and Schlessinger, Cell, 61:203-212, 1990.

Several protein tyrosine kinases are highly expressed in the central nervous system and there is evidence that protein phosphorylation plays a crucial regulatory role in the nervous system. Neurotrophic factors that control the differentiation and maintain the survival of different types of neuronal cells mediate their biological effects by binding to and activating cell surface receptors with intrinsic protein tyrosine kinase activity. Furthermore, protein phosphorylation is a key regulatory mechanism of membrane excitability and ion channel function.

Tyrosine phosphorylation regulates the function of several ion-channels in the central nervous system. Protein kinase C (PKC) can regulate the action of a variety of ion channels including voltage-gated potassium channels, voltage dependent sodium channels as well as the nicotinic acetylcholine receptor. The action of the NMDA receptor can be modulated by protein-tyrosine kinases and phosphatases. Moreover, tyrosine phosphorylation of the nicotine acetylcholine receptors (AChR) increases its rate of desensitization, and may play role in regulation of AChR distribution on the cell membrane. Another example is the delayed rectifier-type K⁺ channel, termed Kv1.2 (also called RAK, RBK2, RCK5 and NGKI). This channel is highly expressed in the brain and cardiac atria, and can be regulated by tyrosine phosphorylation. Tyrosine phosphorylation of Kv1.2 is associated with suppression of Kv1.2- currents. Suppression of Kv1.2 currents was induced by a variety of stimuli including carbachol, bradykinin, PMA and calcium ionophore.

The Ras/MAP kinase signal transduction pathway is highly conserved in evolution and plays an important role in the control of cell growth and differentiation. The MAP kinase signalling pathway in PC12 cells can be activated by NGF, by peptide hormones that activate G-protein coupled receptors, by phorbol ester as well as by calcium influx following membrane depolarization. However, the mechanism underlying activation of the Ras/MAP kinase signaling pathway by G-protein coupled receptors as well as by calcium influx are not known.

Shc is involved in the coupling of both receptor and non-receptor tyrosine kinases to the Ras/MAPK signalling pathways. Overexpression of Shc leads to transformation of 3T3 cells and to neuronal differentiation of PC12 cells. Moreover, Shc induced differentiation of PC12 cells is blocked by a dominant mutant of Ras indicating that Shc acts upstream of Ras. Tyrosine phosphorylated Shc can activate the Ras signaling

pathways by binding to the SH2 domain of the adaptor protein Grb2 that is complexed to the guanine nucleotide releasing factor Sos via its SH3 domains.

Signal transduction pathways that regulate ion channels (e.g., potassium channels and calcium channels) involve G proteins which function as intermediaries between receptors and effectors. Gilman, Ann. Rev. Biochem., 56:615-649 (1987); Brown and Birnbaumer, Ann. Rev. Physiol., 52:197-213 (1990). G-coupled protein receptors are receptors for neurotransmitters, ligands that are responsible for signal production in nerve cells as well as for regulation of proliferation and differentiation of nerves and other cell types. Neurotransmitter receptors exist as different subtypes which are differentially expressed in various tissues and neurotransmitters such as acetylcholine evoke responses throughout the central and peripheral nervous systems. The muscarinic acetylcholine receptors play important roles in a variety of complex neural activities such as learning, memory, arousal and motor and sensory modulation. These receptors have also been implicated in several central nervous system disorders such as Alzheimer's disease, Parkinson's disease, depression and schizophrenia.

Some agents that are involved in a signal transduction pathway regulating one ion channel, for example a potassium channel, may also be involved in one or more other pathways regulating one or more other ion channels, for example a calcium channel. Dolphin, Ann. Rev. Physiol., 52:243-55 (1990); Wilk-Blaszczak et al., Neuron, 12:109-116 (1994). Ion channels may be regulated either with or without a cytosolic second messenger. Hille, Neuron, 9:187-195 (1992). One possible cytosolic second messenger is a tyrosine kinase. Huang et al., Cell, 75:1145-1156 (1993), incorporated herein by reference in its entirety, including any drawings.

The receptors involved in the signal transduction pathways that regulate ion channels are ultimately linked to the ion channels by various intermediate events and agents. For example, such events include an increase in intracellular calcium and inositol triphosphate and production of endothelin. Frucht, et al., Cancer Research, 52:1114-1122 (1992); Schrey, et al., Cancer Research, 52:1786-1790 (1992). Intermediary agents include bombesin, which stimulates DNA synthesis and the phosphorylation of a specific protein kinase C substrate. Rodriguez-Pena, et al., Biochemical and Biophysical Research Communication, 140(1):379-385 (1986); Fisher and Schonbrunn, The Journal of Biological Chemistry, 263(6):2208-2816 (1988).

15 Summary of the Invention

The present invention relates to PYK2 polypeptides, nucleic acids encoding such polypeptides, cells, tissues and animals containing such polypeptides and nucleic acids, antibodies to such polypeptides, assays utilizing such polypeptides, and methods relating to all of the foregoing. PYK2 polypeptides are involved in various signal transduction pathways and thus the present invention provides several agents and methods useful for diagnosing, treating, and preventing various diseases or conditions associated with abnormalities in these pathways.

The present invention is based upon the identification and isolation of a novel non-receptor tyrosine kinase, termed PYK2, that is activated by binding of ligand to G-coupled protein receptors such as bradykinin and acetylcholine. PYK2 has a predicted molecular weight of 111 kD and contains five domains: (1) a relatively long N-terminal domain; (2) a kinase catalytic domain; (3) a proline rich domain; (4) another proline rich domain; and (5) a C-terminal focal adhesion

targeting (FAT) domain. PYK2 does not contain a SH2 or SH3 domain.

The FAT domain of PYK2 has 62% similarity to the FAT domain of another non-receptor tyrosine kinase, FAK, which is also activated by G-coupled proteins. The overall similarity between PYK2 and FAK is 52%. PYK2 is expressed principally in neural tissues, although expression can also be detected in hematopoietic cells at early stages of development and in some tumor cell lines. The expression of PYK2 does not correspond with the expression of FAK.

PYK2 is believed to regulate the activity of potassium channels in response to neurotransmitter signalling. PYK2 enzymatic activity is positively regulated by phosphorylation on tyrosine and results in response to binding of bradykinin, TPA, calcium ionophore, carbachol, TPA + forskolin, and membrane depolarization. The combination of toxins known to positively regulate G-coupled receptor signalling (such as pertussis toxin, cholera toxins, TPA and bradykinin) increases the phosphorylation of PYK2.

Activated PYK2 phosphorylates RAK, a delayed rectifier type potassium channel, and thus suppresses RAK activity. In the same system, FAK does not phosphorylate RAK. PYK2 is responsible for regulating neurotransmitter signalling and thus may be used to treat conditions of nervous system by enhancing or inhibiting such signalling.

Thus, in a first aspect the invention features an isolated, purified, enriched or recombinant nucleic acid encoding a PYK2 polypeptide.

By "isolated" in reference to nucleic acid is meant a polymer of 2 (preferably 21, more preferably 39, most preferably 75) or more nucleotides conjugated to each other, including DNA or RNA that is isolated from a natural source or that is synthesized. The isolated nucleic acid of the present invention is unique in the

sense that it is not found in a pure or separated state in nature. Use of the term "isolated" indicates that a naturally occurring sequence has been removed from its normal cellular environment. Thus, the sequence may be in
5 a cell-free solution or placed in a different cellular environment. The term does not imply that the sequence is the only nucleotide chain present, but does indicate that it is the predominate sequence present (at least 10 - 20% more than any other nucleotide sequence) and is
10 essentially free (about 90 - 95% pure at least) of non-nucleotide material naturally associated with it. Therefore, the term does not encompass an isolated chromosome encoding a PYK2 polypeptide.

By the use of the term "enriched" in reference to
15 nucleic acid is meant that the specific DNA or RNA sequence constitutes a significantly higher fraction (2 - 5 fold) of the total DNA or RNA present in the cells or solution of interest than in normal or diseased cells or in the cells from which the sequence was taken. This
20 could be caused by a person by preferential reduction in the amount of other DNA or RNA present, or by a preferential increase in the amount of the specific DNA or RNA sequence, or by a combination of the two. However, it should be noted that enriched does not imply that there
25 are no other DNA or RNA sequences present, just that the relative amount of the sequence of interest has been significantly increased in a useful manner and preferably separate from a sequence library. The term significant here is used to indicate that the level of increase is
30 useful to the person making such an increase, and generally means an increase relative to other nucleic acids of about at least 2 fold, more preferably at least 5 to 10 fold or even more. The term also does not imply that there is no DNA or RNA from other sources. The other
35 source DNA may, for example, comprise DNA from a yeast or bacterial genome, or a cloning vector such as pUC19. This term distinguishes from naturally occurring events, such

as viral infection, or tumor type growths, in which the level of one mRNA may be naturally increased relative to other species of mRNA. That is, the term is meant to cover only those situations in which a person has
5 intervened to elevate the proportion of the desired nucleic acid.

It is also advantageous for some purposes that a nucleotide sequence be in purified form. The term "purified" in reference to nucleic acid does not require
10 absolute purity (such as a homogeneous preparation); instead, it represents an indication that the sequence is relatively purer than in the natural environment (compared to the natural level this level should be at least 2-5 fold greater, e.g., in terms of mg/ml). Individual clones
15 isolated from a cDNA library may be purified to electrophoretic homogeneity. The claimed DNA molecules obtained from these clones could be obtained directly from total DNA or from total RNA. The cDNA clones are not naturally occurring, but rather are preferably obtained
20 via manipulation of a partially purified naturally occurring substance (messenger RNA). The construction of a cDNA library from mRNA involves the creation of a synthetic substance (cDNA) and pure individual cDNA clones can be isolated from the synthetic library by clonal
25 selection of the cells carrying the cDNA library. Thus, the process which includes the construction of a cDNA library from mRNA and isolation of distinct cDNA clones yields an approximately 10^6 -fold purification of the native message. Thus, purification of at least one order of
30 magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly contemplated.

By "a PYK2 polypeptide" is meant two or more contiguous amino acids set forth in the full length amino
35 acid sequence of SEQ ID NO:2. The PYK2 polypeptide can be encoded by a full-length nucleic acid sequence or any portion of the full-length nucleic acid sequence, so long

as a functional activity of the polypeptide is retained. Preferred functional activities include the ability to phosphorylate and regulate RAK and/or other potassium channels.

5 In preferred embodiments the isolated nucleic acid comprises, consists essentially of, or consists of a nucleic acid sequence set forth in the full length nucleic acid sequence SEQ ID NO:1 or at least 27, 30, 35, 40 or 50 contiguous nucleotides thereof and the PYK2 polypeptide
10 comprises, consists essentially of, or consists of at least 9, 10, 15, 20, or 30 contiguous amino acids of a PYK2 polypeptide.

By "comprising" it is meant including, but not limited to, whatever follows the word "comprising". Thus,
15 use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the
20 phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or
25 contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending
30 upon whether or not they affect the activity or action of the listed elements.

Compositions and probes of the present invention may contain human nucleic acid encoding a PYK-2 polypeptide but are substantially free of nucleic acid not
35 encoding a human PYK-2 polypeptide. The human nucleic acid encoding a PYK-2 polypeptide is at least 18 contiguous bases of the nucleotide sequence set forth in

SEQ. ID NO. 1 and will selectively hybridize to human genomic DNA encoding a PYK-2 polypeptide, or is complementary to such a sequence. The nucleic acid may be isolated from a natural source by cDNA cloning or
5 subtractive hybridization; the natural source may be blood, semen, and tissue of various organisms including eukaryotes, mammals, birds, fish, plants, gorillas, rhesus monkeys, chimpanzees and humans; and the nucleic acid may be synthesized by the triester method or by using an
10 automated DNA synthesizer. In yet other preferred embodiments the nucleic acid is a conserved or unique region, for example those useful for the design of hybridization probes to facilitate identification and cloning of additional polypeptides, the design of PCR
15 probes to facilitate cloning of additional polypeptides, and obtaining antibodies to polypeptide regions.

By "conserved nucleic acid regions", are meant regions present on two or more nucleic acids encoding a PYK2 polypeptide, to which a particular nucleic acid
20 sequence can hybridize to under lower stringency conditions. Examples of lower stringency conditions suitable for screening for nucleic acid encoding PYK2 polypeptides are provided in Abe, et al. J. Biol. Chem., 19:13361 (1992) (hereby incorporated by reference herein in its
25 entirety, including any drawings). Preferably, conserved regions differ by no more than 7 out of 20 nucleotides.

By "unique nucleic acid region" is meant a sequence present in a full length nucleic acid coding for a PYK2 polypeptide that is not present in a sequence
30 coding for any other naturally occurring polypeptide. Such regions preferably comprise 12 or 20 contiguous nucleotides present in the full length nucleic acid encoding a PYK2 polypeptide.

The invention also features a nucleic acid probe
35 for the detection of a PYK2 polypeptide or nucleic acid encoding a PYK2 polypeptide in a sample. The nucleic acid

probe contains nucleic acid that will hybridize to a sequence set forth in SEQ ID NO:1.

In preferred embodiments the nucleic acid probe hybridizes to nucleic acid encoding at least 12, 27, 30, 5 35, 40 or 50 contiguous amino acids of the full-length sequence set forth in SEQ ID NO:2. Various low or high stringency hybridization conditions may be used depending upon the specificity and selectivity desired.

By "high stringency hybridization conditions" is 10 meant those hybridizing conditions that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% SDS at 50°C; (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide 15 with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M Sodium pyrophosphate, 5 x Denhardt's solution, sonicated 20 salmon sperm DNA (50 g/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS. Under stringent hybridization conditions only highly complementary nucleic acid sequences hybridize. Preferably, such conditions prevent hybridization of 25 nucleic acids having 1 or 2 mismatches out of 20 contiguous nucleotides.

Methods for using the probes include detecting the presence or amount PYK2 RNA in a sample by contacting the sample with a nucleic acid probe under conditions such 30 that hybridization occurs and detecting the presence or amount of the probe bound to PYK2 RNA. The nucleic acid duplex formed between the probe and a nucleic acid sequence coding for a PYK2 polypeptide may be used in the identification of the sequence of the nucleic acid 35 detected (for example see, Nelson et al., in Nonisotopic DNA Probe Techniques, p. 275 Academic Press, San Diego (Kricka, ed., 1992) hereby incorporated by reference

herein in its entirety, including any drawings). Kits for performing such methods may be constructed to include a container means having disposed therein a nucleic acid probe.

5 The invention also features recombinant nucleic acid, preferably in a cell or an organism. The recombinant nucleic acid may contain a sequence set forth in SEQ ID NO:1 and a vector or a promoter effective to initiate transcription in a host cell. The recombinant
10 nucleic acid can alternatively contain a transcriptional initiation region functional in a cell, a sequence complimentary to an RNA sequence encoding a PYK2 polypeptide and a transcriptional termination region functional in a cell.

15 In another aspect the invention features an isolated, enriched or purified PYK2 polypeptide.

 By "isolated" in reference to a polypeptide is meant a polymer of 2 (preferably 7, more preferably 13, most preferably 25) or more amino acids conjugated to each
20 other, including polypeptides that are isolated from a natural source or that are synthesized. The isolated polypeptides of the present invention are unique in the sense that they are not found in a pure or separated state in nature. Use of the term "isolated" indicates that a
25 naturally occurring sequence has been removed from its normal cellular environment. Thus, the sequence may be in a cell-free solution or placed in a different cellular environment. The term does not imply that the sequence is the only amino acid chain present, but that it is the
30 predominate sequence present (at least 10 - 20% more than any other sequence) and is essentially free (about 90 - 95% pure at least) of non-amino acid material naturally associated with it.

 By the use of the term "enriched" in reference to
35 a polypeptide is meant that the specific amino acid sequence constitutes a significantly higher fraction (2 - 5 fold) of the total of amino acids present in the cells

or solution of interest than in normal or diseased cells or in the cells from which the sequence was taken. This could be caused by a person by preferential reduction in the amount of other amino acids present, or by a
5 preferential increase in the amount of the specific amino acid sequence of interest, or by a combination of the two. However, it should be noted that enriched does not imply that there are no other amino acid sequences present, just that the relative amount of the sequence of interest has
10 been significantly increased. The term significant here is used to indicate that the level of increase is useful to the person making such an increase, and generally means an increase relative to other amino acids of about at least 2 fold, more preferably at least 5 to 10 fold or
15 even more. The term also does not imply that there is no amino acid from other sources. The other source amino acid may, for example, comprise amino acid encoded by a yeast or bacterial genome, or a cloning vector such as pUC19. The term is meant to cover only those situations
20 in which man has intervened to elevate the proportion of the desired amino acid.

It is also advantageous for some purposes that an amino acid sequence be in purified form. The term "purified" in reference to a polypeptide does not require
25 absolute purity (such as a homogeneous preparation); instead, it represents an indication that the sequence is relatively purer than in the natural environment (compared to the natural level this level should be at least 2-5 fold greater, e.g., in terms of mg/ml). Purification of
30 at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly contemplated. The substance is preferably free of contamination at a functionally significant level, for example 90%, 95%, or 99% pure.

35 In preferred embodiments the FYK-2 polypeptide contains at least 9, 10, 15, 20, or 30 contiguous amino

acids of the full-length sequence set forth in SEQ ID NO:2.

In yet another aspect the invention features a purified antibody (e.g., a monoclonal or polyclonal
5 antibody) having specific binding affinity to a PYK2 polypeptide. The antibody contains a sequence of amino acids that is able to specifically bind to a PYK2 polypeptide.

By "specific binding affinity" is meant that the
10 antibody will bind to a PYK-2 polypeptide at a certain detectable amount but will not bind other polypeptides, such as FAK polypeptides to the same extent, under identical conditions.

Antibodies having specific binding affinity to a
15 PYK2 polypeptide may be used in methods for detecting the presence and/or amount of a PYK2 polypeptide in a sample by contacting the sample with the antibody under conditions such that an immunocomplex forms and detecting the presence and/or amount of the antibody conjugated to
20 the PYK2 polypeptide. Diagnostic kits for performing such methods may be constructed to include a first container means containing the antibody and a second container means having a conjugate of a binding partner of the antibody and a label.

25 In another aspect the invention features a hybridoma which produces an antibody having specific binding affinity to a PYK2 polypeptide.

By "hybridoma" is meant an immortalized cell line which is capable of secreting an antibody, for example a
30 PYK2 antibody.

In preferred embodiments the PYK2 antibody comprises a sequence of amino acids that is able to specifically bind a PYK2 polypeptide.

Another aspect of the invention features a method
35 of detecting the presence or amount of a compound capable of binding to a PYK2 polypeptide. The method involves incubating the compound with a PYK2 polypeptide and

detecting the presence or amount of the compound bound to the PYK2 polypeptide.

In preferred embodiments, the compound inhibits a phosphorylation activity of PYK2 and is selected from the group consisting of tyrphostins, quinazolines, quinaxolines, and quinolines. The present invention also features compounds capable of binding and inhibiting PYK2 polypeptide that are identified by methods described above.

10 In another aspect the invention features a method of screening potential agents useful for treatment of a disease or condition characterized by an abnormality in a signal transduction pathway that contains an interaction between a PYK2 polypeptide and a natural binding partner
15 (NBP). The method involves assaying potential agents for those able to promote or disrupt the interaction as an indication of a useful agent.

By "screening" is meant investigating an organism for the presence or absence of a property. The process
20 may include measuring or detecting various properties, including the level of signal transduction and the level of interaction between a PYK2 polypeptide and a NBP.

By "disease or condition" is meant a state in an organism, e.g., a human, which is recognized as abnormal
25 by members of the medical community. The disease or condition may be characterized by an abnormality in one or more signal transduction pathways in a cell, preferably a cell listed in table 1, wherein one of the components of the signal transduction pathway is either a PYK2
30 polypeptide or a NBP.

Specific diseases or disorders which might be treated or prevented, based upon the affected cells include: myasthenia gravis; neuroblastoma; disorders caused by neuronal toxins such as cholera toxin, pertussis
35 toxin, or snake venom; acute megakaryocytic myelosis; thrombocytopenia; those of the central nervous system such as seizures, stroke, head trauma, spinal cord injury,

- hypoxia-induced nerve cell damage such as in cardiac arrest or neonatal distress, epilepsy, neurodegenerative diseases such as Alzheimer's disease, Huntington's disease and Parkinson's disease, dementia, muscle tension, depression, anxiety, panic disorder, obsessive-compulsive disorder, post-traumatic stress disorder, schizophrenia, neuroleptic malignant syndrome, and Tourette's syndrome. Conditions that may be treated by PYK2 inhibitors include epilepsy, schizophrenia, extreme hyperactivity in children, chronic pain, and acute pain. Examples of conditions that may be treated by PYK2 enhancers (for example a phosphatase inhibitor) include stroke, Alzheimer's, Parkinson's, other neurodegenerative diseases and migraine.
- Preferred disorders include epilepsy, stroke, schizophrenia, and Parkinson's disorder as there is an established relationship between these disorders and the function of potassium channels. See, McLean et al., Epilepsia 35:S5-S9 1994; Ricard-Mousnier et al., Neurophysiologie Clinique 23:395-421, 1993; Crit. Rev. Neurobiol. 7:187-203, 1994; Simon and Lin, Biophys. J. 64:A100, 1993; Birnstiel et al., Synapse (NY) 11:191-196, 1992; Coleman et al., Brain Res. 575:138-142 1992; Popolitz et al., Br. J. Pharmacol. 104:907-913, 1991; Murphy et al., Exp. Brain Res. 84:355-358, 1991; Rutecki et al., Epilepsia 32:1-2, 1991; Fisher and Coyle (ed), Frontiers of Clinical Neuroscience, Vol. 11 "Neurotransmitters and Epilepsy"; Meeting, Woods Hole MA, USA IX+260P. John Wiley and Sons, Inc. NY, NY; Treherne and Ashford, Neuroscience 40:523-532, 1991; Gehlert, Prog. Neuro-Psychopharmacol. Biol. Psychiatry 18:1093-1102, 1994; Baudy, Expert Opin Ther. Pat. 1994 4/4:343-378; Porter and Rogawski, Epilepsia 33:S1-S6, 1992; Murphy, J. Physiol. 453:167-183, 1992; Cromakalim, Drugs Future 17/3:237-239, 1992; Carmeliet, Eur. Heart J. 12:30-37, 1991; Olpe et al., Experientia 47/3:254-257, 1991; Andrade et al., Science

234/4781:1261-1265, 1986; Forster, J. Neurosci. Methods
13/3-4:199-212, 1985.

In preferred embodiments, the methods described herein involve identifying a patient in need of treatment.

5 Those skilled in the art will recognize that various techniques may be used to identify such patients. For example, cellular potassium levels may be measured or the individuals genes may be examined for a defect.

By "abnormality" is meant an a level which is
10 statistically different from the level observed in organisms not suffering from such a disease or condition and may be characterized as either an excess amount, intensity or duration of signal or a deficient amount, intensity or duration of signal. The abnormality in signal
15 transduction may be realized as an abnormality in cell function, viability or differentiation state. We have determined that such abnormality in a pathway can be alleviated by action at the PYK2:NBP interaction site in the pathway. An abnormal interaction level may also
20 either be greater or less than the normal level and may impair the normal performance or function of the organism. Thus, it is also possible to screen for agents that will be useful for treating a disease or condition, characterized by an abnormality in the signal transduction pathway,
25 by testing compounds for their ability to affect the interaction between a PYK2 polypeptide and a NBP, since the complex formed by such interaction is part of the signal transduction pathway. However, the disease or condition may be characterized by an abnormality in the
30 signal transduction pathway even if the level of interaction between the PYK2 polypeptide and NBP is normal.

By "interact" is meant any physical association between polypeptides, whether covalent or non-covalent.
35 This linkage can include many chemical mechanisms, for instance covalent binding, affinity binding, intercalation, coordinate binding and complexation.

Examples of non-covalent bonds include electrostatic bonds, hydrogen bonds, and Van der Waals bonds. Furthermore, the interactions between polypeptides may either be direct or indirect. Thus, the association
5 between two given polypeptides may be achieved with an intermediary agent, or several such agents, that connects the two proteins of interest (e.g., a PYK2 polypeptide and a NBP). Another example of an indirect interaction is the independent production, stimulation, or inhibition of both
10 a PYK2 polypeptide and NBP by a regulatory agent. Depending upon the type of interaction present, various methods may be used to measure the level of interaction. For example, the strengths of covalent bonds are often measured in terms of the energy required to break a
15 certain number of bonds (i.e., kcal/mol). Non-covalent interactions are often described as above, and also in terms of the distance between the interacting molecules. Indirect interactions may be described in a number of ways, including the number of intermediary agents
20 involved, or the degree of control exercised over the PYK2 polypeptide relative to the control exercised over the NBP.

By "disrupt" is meant that the interaction between the PYK2 polypeptide and NBP is reduced either by
25 preventing expression of the PYK2 polypeptide, or by preventing expression of the NBP, or by specifically preventing interaction of the naturally synthesized proteins or by interfering with the interaction of the proteins.

30 By "promote" is meant that the interaction between a PYK2 polypeptide and NBP is increased either by increasing expression of a PYK2 polypeptide, or by increasing expression of a NBP, or by decreasing the dephosphorylating activity of the corresponding regulatory
35 TP (or other phosphatase acting on other phosphorylated signalling components) by promoting interaction of the PYK2 polypeptide and NBP or by prolonging the duration of

the interaction. Covalent binding can be promoted either by direct condensation of existing side chains or by the incorporation of external bridging molecules. Many bivalent or polyvalent linking agents are useful in coupling polypeptides, such as an antibody, to other molecules. For example, representative coupling agents can include organic compounds such as thioesters, carbodiimides, succinimide esters, diisocyanates, glutaraldehydes, diazobenzenes and hexamethylene diamines. This listing is not intended to be exhaustive of the various classes of coupling agents known in the art but, rather, is exemplary of the more common coupling agents. (See Killen and Lindstrom 1984, J. Immunol. 133:1335-2549; Jansen, F.K., et al., 1982, Immunological Rev. 62:185-216; and Vitetta et al., supra).

By "NBP" is meant a natural binding partner of a PYK2 polypeptide that naturally associates with a PYK2 polypeptide. The structure (primary, secondary, or tertiary) of the particular natural binding partner will influence the particular type of interaction between the PYK2 polypeptide and the natural binding partner. For example, if the natural binding partner comprises a sequence of amino acids complementary to the PYK2 polypeptide, covalent bonding may be a possible interaction. Similarly, other structural characteristics may allow for other corresponding interactions. The interaction is not limited to particular residues and specifically may involve phosphotyrosine, phosphoserine, or phosphothreonine residues. A broad range of sequences may be capable of interacting with PYK2 polypeptides. Using techniques well known in the art, one may identify several natural binding partners for PYK2 polypeptides. Examples of PYK-2 natural binding partners include Grb-2 and Sos1.

By "signal transduction pathway" is meant the sequence of events that involves the transmission of a message from an extracellular protein to the cytoplasm

through a cell membrane. The signal ultimately will cause the cell to perform a particular function, for example, to uncontrollably proliferate and therefore cause cancer. Various mechanisms for the signal transduction pathway
5 (Fry et al., Protein Science, 2:1785-1797, 1993) provide possible methods for measuring the amount or intensity of a given signal. Depending upon the particular disease associated with the abnormality in a signal transduction pathway, various symptoms may be detected. Those skilled
10 in the art recognize those symptoms that are associated with the various other diseases described herein. Furthermore, since some adapter molecules recruit secondary signal transducer proteins towards the membrane, one measure of signal transduction is the concentration
15 and localization of various proteins and complexes. In addition, conformational changes that are involved in the transmission of a signal may be observed using circular dichroism and fluorescence studies.

In preferred embodiments the screening method
20 involves growing cells (*i.e.*, in a dish) that either naturally or recombinantly express a G-coupled protein receptor, PYK2, and RAK. The test compound is added at a concentration from 0.1 μ M to 100 μ M and the mixture is incubated from 5 minutes to 2 hours. The ligand is added
25 to the G-coupled protein receptor for preferably 5 to 30 minutes and the cells are lysed. RAK is isolated using immunoprecipitation or ELISA by binding to a specific monoclonal antibody. The amount of phosphorylation compared to cells that were not exposed to a test compound
30 is measured using an anti-phosphotyrosine antibody (preferably polyclonal). Examples of compounds that could be tested in such screening methods include tyrphostins, quinazolines, quinoxolines, and quinolines.

The quinazolines, tyrphostins, quinolines, and
35 quinoxolines referred to above include well known compounds such as those described in the literature. For example, representative publications describing

quinazoline include Barker et al., EPO Publication No. 0 520 722 A1; Jones et al., U.S. Patent No. 4,447,608; Kabbe et al., U.S. Patent No. 4,757,072; Kaul and Vougioukas, U.S. Patent No. 5, 316,553; Kreighbaum and
5 Comer, U.S. Patent No. 4,343,940; Pegg and Wardleworth, EPO Publication No. 0 562 734 A1; Barker et al., Proc. of Am. Assoc. for Cancer Research 32:327 (1991); Bertino, J.R., Cancer Research 3:293-304 (1979); Bertino, J.R., Cancer Research 9(2 part 1):293-304 (1979); Curtin et al.,
10 Br. J. Cancer 53:361-368 (1986); Fernandes et al., Cancer Research 43:1117-1123 (1983); Ferris et al., J. Org. Chem. 44(2):173-178; Fry et al., Science 265:1093-1095 (1994); Jackman et al., Cancer Research 51:5579-5586 (1981); Jones et al., J. Med. Chem. 29(6):1114-1118; Lee and Skibo,
15 Biochemistry 26(23):7355-7362 (1987); Lemus et al., J. Org. Chem. 54:3511-3518 (1989); Ley and Seng, Synthesis 1975:415-522 (1975); Maxwell et al., Magnetic Resonance in Medicine 17:189-196 (1991); Mini et al., Cancer Research 45:325-330 (1985); Phillips and Castle, J. Heterocyclic
20 Chem. 17(19):1489-1596 (1980); Reece et al., Cancer Research 47(11):2996-2999 (1977); Sculier et al., Cancer Immunol. and Immunother. 23:A65 (1986); Sikora et al., Cancer Letters 23:289-295 (1984); Sikora et al., Analytical Biochem. 172:344-355 (1988); all of which are
25 incorporated herein by reference in their entirety, including any drawings.

Quinoxaline is described in Kaul and Vougioukas, U.S. Patent No. 5,316,553, incorporated herein by reference in its entirety, including any drawings.

30 Quinolines are described in Dolle et al., J. Med. Chem. 37:2627-2629 (1994); MaGuire, J. Med. Chem. 37:2129-2131 (1994); Burke et al., J. Med. Chem. 36:425-432 (1993); and Burke et al., BioOrganic Med. Chem. Letters 2:1771-1774 (1992), all of which are incorporated by
35 reference in their entirety, including any drawings.

Tyrphostins are described in Allen et al., Clin. Exp. Immunol. 91:141-156 (1993); Anafi et al., Blood

- 82:12:3524-3529 (1993); Baker et al., J. Cell Sci. 102:543-555 (1992); Bilder et al., Amer. Physiol. Soc. pp. 6363-6143:C721-C730 (1991); Brunton et al., Proceedings of Amer. Assoc. Cancer Rsch. 33:558 (1992);
- 5 Bryckaert et al., Experimental Cell Research 199:255-261 (1992); Dong et al., J. Leukocyte Biology 53:53-60 (1993); Dong et al., J. Immunol. 151(5):2717-2724 (1993); Gazit et al., J. Med. Chem. 32:2344-2352 (1989); Gazit et al., " J. Med. Chem. 36:3556-3564 (1993); Kaur et al., Anti-
- 10 Cancer Drugs 5:213-222 (1994); Kaur et al., King et al., Biochem. J. 275:413-418 (1991); Kuo et al., Cancer Letters 74:197-202 (1993); Levitzki, A., The FASEB J. 6:3275-3282 (1992); Lyall et al., J. Biol. Chem. 264:14503-14509 (1989); Peterson et al., The Prostate 22:335-345 (1993);
- 15 Pillemer et al., Int. J. Cancer 50:80-85 (1992); Posner et al., Molecular Pharmacology 45:673-683 (1993); Rendu et al., Biol. Pharmacology 44(5):881-888 (1992); Sauro and Thomas, Life Sciences 53:371-376 (1993); Sauro and Thomas, J. Pharm. and Experimental Therapeutics 267(3):119-1125
- 20 (1993); Wolbring et al., J. Biol. Chem. 269(36):22470-22472 (1994); and Yoneda et al., Cancer Research 51:4430-4435 (1991); all of which are incorporated herein by reference in their entirety, including any drawings.

In another aspect the invention features a method

25 of diagnosis of an organism for a disease or condition characterized by an abnormality in a signal transduction pathway that contains an interaction between a PYK2 polypeptide and a NBP. The method involves detecting the level of interaction as an indication of said disease or

30 condition.

By "organism" is meant any living creature. The term includes mammals, and specifically humans. Preferred organisms include mice, as the ability to treat or diagnose mice is often predictive of the ability to function in other organisms such as humans.

35

By "diagnosis" is meant any method of identifying a symptom normally associated with a given disease or

condition. Thus, an initial diagnosis may be conclusively established as correct by the use of additional confirmatory evidence such as the presence of other symptoms. Current classification of various diseases and conditions is constantly changing as more is learned about the mechanisms causing the diseases or conditions. Thus, the detection of an important symptom, such as the detection of an abnormal level of interaction between PYK2 polypeptides and NBPs may form the basis to define and diagnose a newly named disease or condition. For example, conventional cancers are classified according to the presence of a particular set of symptoms. However, a subset of these symptoms may both be associated with an abnormality in a particular signalling pathway, such as the ras²¹ pathway and in the future these diseases may be reclassified as ras²¹ pathway diseases regardless of the particular symptoms observed.

Yet another aspect of the invention features a method for treatment of an organism having a disease or condition characterized by an abnormality in a signal transduction pathway. The signal transduction pathway contains an interaction between a PYK2 polypeptide and a NBP and the method involves promoting or disrupting the interaction, including methods that target the PYK2:NBP interaction directly, as well as methods that target other points along the pathway.

In preferred embodiments the signal transduction pathway regulates an ion channel, for example, a potassium ion, the disease or condition which is diagnosed or treated are those described above, the agent is a dominant negative mutant protein provided by gene therapy or other equivalent methods as described below and the agents is therapeutically effective and has an EC₅₀ or IC₅₀ as described below.

By "dominant negative mutant protein" is meant a mutant protein that interferes with the normal signal transduction pathway. The dominant negative mutant

protein contains the domain of interest (e.g., an PYK2 polypeptide or a NBP), but has a mutation preventing proper signaling, for example by preventing binding of a second domain from the same protein. One example of a dominant negative protein is described in Millauer et al., Nature February 10, 1994. The agent is preferably a peptide which blocks or promotes interaction of the PYK2 polypeptide and the NBP. The peptide may be recombinant, purified, or placed in a pharmaceutically acceptable carrier or diluent.

An EC_{50} or IC_{50} of less than or equal to 100 μM is preferable, and even more preferably less than or equal to 50 μM , and most preferably less than or equal to 20 μM . Such lower EC_{50} 's or IC_{50} 's are advantageous since they allow lower concentrations of molecules to be used in vivo or in vitro for therapy or diagnosis. The discovery of molecules with such low EC_{50} 's and IC_{50} 's enables the design and synthesis of additional molecules having similar potency and effectiveness. In addition, the molecule may have an EC_{50} or IC_{50} less than or equal to 100 μM at one or more, but not all cells chosen from the group consisting of parathyroid cell, bone osteoclast, juxtaglomerular kidney cell, proximal tubule kidney cell, distal tubule kidney cell, cell of the thick ascending limb of Henle's loop and/or collecting duct, central nervous system cell, keratinocyte in the epidermis, parafollicular cell in the thyroid (C-cell), intestinal cell, trophoblast in the placenta, platelet, vascular smooth muscle cell, cardiac atrial cell, gastrin-secreting cell, glucagon-secreting cell, kidney mesangial cell, mammary cell, beta cell, fat/adipose cell, immune cell and GI tract cell.

By "therapeutically effective amount" is meant an amount of a pharmaceutical composition having a therapeutically relevant effect. A therapeutically relevant effect relieves to some extent one or more symptoms of the disease or condition in the patient; or returns to normal either partially or completely one or

more physiological or biochemical parameters associated with or causative of the disease or condition. Generally, a therapeutically effective amount is between about 1 nmole and 1 μ mole of the molecule, depending on its EC_{50} or
5 IC_{50} and on the age and size of the patient, and the disease associated with the patient.

In other aspects, the invention provides transgenic, nonhuman mammals containing a transgene encoding a PYK2 polypeptide or a gene effecting the
10 expression of a PYK2 polypeptide. Such transgenic nonhuman mammals are particularly useful as an *in vivo* test system for studying the effects of introducing a PYK2 polypeptide, regulating the expression of a PYK2 polypeptide (*i.e.*, through the introduction of additional
15 genes, antisense nucleic acids, or ribozymes).

A "transgenic animal" is an animal having cells that contain DNA which has been artificially inserted into a cell, which DNA becomes part of the genome of the animal which develops from that cell. Preferred transgenic
20 animals are primates, mice, rats, cows, pigs, horses, goats, sheep, dogs and cats. The transgenic DNA may encode for a human PYK2 polypeptide. Native expression in an animal may be reduced by providing an amount of anti-sense RNA or DNA effective to reduce expression of the
25 receptor.

In another aspect, the invention describes a polypeptide comprising a recombinant PYK2 polypeptide or a unique fragment thereof. By "unique fragment," is meant an amino acid sequence present in a full-length PYK2
30 polypeptide that is not present in any other naturally occurring polypeptide. Preferably, such a sequence comprises 6 contiguous amino acids present in the full sequence. More preferably, such a sequence comprises 12 contiguous amino acids present in the full sequence. Even
35 more preferably, such a sequence comprises 18 contiguous amino acids present in the full sequence.

By "recombinant PYK2 polypeptide" is meant to include a polypeptide produced by recombinant DNA techniques such that it is distinct from a naturally occurring polypeptide either in its location (e.g.,
5 present in a different cell or tissue than found in nature), purity or structure. Generally, such a recombinant polypeptide will be present in a cell in an amount different from that normally observed in nature.

In another aspect, the invention describes a
10 recombinant cell or tissue containing a purified nucleic acid coding for a PYK2 polypeptide. In such cells, the nucleic acid may be under the control of its genomic regulatory elements, or may be under the control of exogenous regulatory elements including an exogenous
15 promoter. By "exogenous" it is meant a promoter that is not normally coupled *in vivo* transcriptionally to the coding sequence for the PYK2 polypeptide.

In another aspect, the invention features a PYK2 polypeptide binding agent able to bind to a PYK2
20 polypeptide. The binding agent is preferably a purified antibody which recognizes an epitope present on a PYK2 polypeptide. Other binding agents include molecules which bind to the PYK2 polypeptide and analogous molecules which bind to a PYK2 polypeptide.

25 By "purified" in reference to an antibody is meant that the antibody is distinct from naturally occurring antibody, such as in a purified form. Preferably, the antibody is provided as a homogeneous preparation by standard techniques. Uses of antibodies to
30 the cloned polypeptide include those to be used as therapeutics, or as diagnostic tools.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Figures and Tables

Figure 1 shows a schematic representation of the PYK2 domains (including a kinase domain, a proline rich domain, and a Fat domain) and potential binding sites (including YAEI, YLNV, and YVVV).

Figure 2 shows a possible mechanism for the membrane depolarization and calcium influx that stimulate MEK and MAP kinase via activation of Ras. In PC12 cells, membrane depolarization leads to calcium influx through L-type calcium channels and activates MAP kinase. Calcium influx leads to activation of Ras and the activation of MAP in response to calcium influx is inhibited by a dominant negative mutant of Ras. Elevation of intracellular calcium concentration by various stimuli leads to the activation of PYK2. PYK2 recruits Shc/Grb2/Sos complex leading to the activation of a signaling pathway composed of Ras, Raf, MAPKK, MAPK to relay signals to the cell nucleus.

Figure 3 shows a model for the extracellular stimuli that activate PYK2 and potential target molecule that is tyrosine phosphorylated in response to PYK2 activation. The tyrosine kinase activity of PYK2 is activated by a variety of extracellular signals that stimulate calcium influx including activation of the nicotinic acetylcholine receptor by carbachol, membrane depolarization by KCl (75mM), and treatment with a calcium ionophore. Activation of PYK2 by these stimuli requires the presence of extracellular calcium. PYK2 is also stimulated in response to bradykinin (BK) induced activation of its G-protein coupled receptor leading to PI hydrolysis and Ca^{+2} release from internal stores. PYK2 is also activated in response to phorbol ester (PMA) treatment that binds to and activates several PKC isozymes. Co-expression experiments in transfected cells and in frog oocytes show that activation of PYK2 leads to tyrosine phosphorylation (thick arrow) of the delayed

rectifier-type K⁺ channel Kv1.2 and to suppression of Kv1.2 channel mediated currents.

Figure 4 shows an alignment of PYK-2 amino acids to those of 4 other proteins, Fak, Fer, HER4 and AB1.

5 Table 1 shows the expression pattern and levels of PYK2 in various cell lines as checked by multiple methods.

Description of the Preferred Embodiments

The present invention relates to PYK2
10 polypeptides, nucleic acids encoding such polypeptides; cells, tissues and animals containing such nucleic acids, antibodies to such polypeptides, assays utilizing such polypeptides, and methods relating to all of the foregoing. Those skilled in the art will recognize that
15 many of the methods described below in relation to PYK-2, a NBP, or a complex of PYK-2 and a NBP could also be utilized with respect to the other members of this group.

We describe the isolation and characterization of a novel non-receptor tyrosine kinase termed PYK2, that is
20 highly expressed in the nervous system and in the adult rat brain. PYK2 is a second member of Fak family of non-receptor protein tyrosine kinases. However, PYK2 exhibits diffuse cytoplasmic localization unlike the preferential localization of Fak in focal adhesion areas.

25 The examples presented herein reveal a novel mechanism for the coupling, between G-protein coupled receptors and the MAP kinase signaling pathway. We also show that calcium influx induced by membrane depolarization following activation of the nicotinic
30 acetylcholine receptor or other stimuli that cause calcium influx lead to the activation of PYK2, tyrosine phosphorylation of Shc, recruitment of Grb2/Sos and activation of the MAP kinase signaling pathway.

PYK2 is activated by extracellular signals that
35 lead to calcium influx or calcium release from internal stores. PYK2 is phosphorylated on tyrosine residues in

response to a variety of external stimuli that cause membrane depolarization and Ca^{+2} influx such as the activation of the nicotinic acetylcholine receptor. Tyrosine phosphorylation of PYK2 is also stimulated by the
5 neuropeptide Bradykinin that activates a G-protein coupled receptor as well as by Phorbol myristate acetate (PMA). Experiments in transfected cells and in *Xenopus* oocytes, microinjected with PYK2 mRNA, indicate that activation of PYK2 can lead to tyrosine phosphorylation of a delayed
10 rectifier-type potassium channel protein and to suppression of potassium currents via this channel. These results suggest a novel mechanism by which a non-receptor tyrosine kinases, in the nervous system, can be both activated by and can modulate the action of ion-channel
15 proteins.

Activation of PYK2 in PC12 cells by the same stimuli leads to the recruitment of Shc/Grb2/Sos complex and to the activation of the MAP kinase signaling pathway that relays signals to the cell nucleus. The experiments
20 presented thus show that PYK2 may also provide a link between G-protein coupled receptors and calcium influx and the MAP kinase signaling pathway; a pathway that relays signals from the cell surface to regulate transcriptional events in the nucleus. Overexpression of PYK2 leads to
25 activation of MAP kinase. Moreover, the effects of PYK2 on tyrosine phosphorylation and action of the Kv1.2 potassium channel reveals a novel mechanism for heterologous regulation of ion-channel function by activation of an intermediate protein tyrosine kinase.
30 PYK2 can, therefore, couple neuropeptide hormones that act via G-protein coupled receptors that stimulate phosphotydyinositot hydrolysis and the action of target channel molecules.

Transient co-expression experiments of PYK2 with
35 the delayed rectifier K^{+} channel Kv1.2 show that the channel protein undergoes tyrosine phosphorylation in response to PYK2 activation. Moreover, currents exhibited

by Kv1.2 channel expressed in frog oocytes were blocked by co-expression of the PYK2 protein. However, co-expression of a kinase negative mutant of PYK2 released PMA induced suppression of Kv1.2 currents. PYK2 activation may
5 provide a rapid and highly localized control mechanism for ion channel function and kinase activation induced by neuronal stimuli that elevate intracellular calcium leading, to neuronal integration and synaptic efficacy.

These results reveal a role for PYK2 in
10 activation of the MAP kinase signaling pathway by ion channels, calcium influx and G-protein coupled receptors in PC12 cells and may provide a mechanism for signal transduction induced by these stimuli in the nervous system. Furthermore, tyrosine phosphorylation of Shc in
15 response to membrane depolarization and carbachol treatment was dependent on the presence of extracellular calcium, indicating that calcium-influx plays a role in regulation of Shc phosphorylation by these stimuli.

Similarly, PYK2 may modulate the action of ion
20 channels that mediate their responses via and are sensitive to intracellular calcium concentration. PYK2 may therefore provide an autoregulatory role for the very same channel responsible for PYK2 activation. A potential target of PYK2 is the nicotinic acetylcholine receptor.
25 Activation of the nicotinic acetylcholine receptor in PC12 cells leads to strong and rapid tyrosine phosphorylation of PYK2.

The nicotinic acetylcholine receptor is subject to gylation can modulate the activity of the tyrosine
30 phosphorylation. Tyrosine phosphorylation of Shc in response to carbachol treatment is induced via stimulation of the nicotinic acetylcholine receptor as determined by pharmacological analysis. The nicotinic agonist DMPP induced phosphorylation of Shc, whereas muscarine had no
35 effect, the nicotinic antagonist mecamylamine blocked the effect of carbachol, whereas the muscarinic antagonist atropine had no effect. The effect of carbachol on

tyrosine phosphorylation of Shc was transient with maximum tyrosine phosphorylation detected after one minute followed by a rapid decline. NGF however, induced persistent stimulation of Shc phosphorylation for as long as five hours after the addition of NGF. The duration of Shc phosphorylation may have an important impact on the Ras signaling pathway and gene expression induced by these stimuli.

The model presented herein may represent the mechanism underlying calcium mediated regulation of gene expression in neuronal cells induced by MMDA receptor or voltage sensitive calcium channels. The expression pattern of PYK2, the external stimuli that activate this kinase together with its role in the control of MAP kinase signaling pathway suggests a potential role for PYK2 in the control of a broad array of processes in the central nervous system including neuronal plasticity. in the nervous svstem.

Since PYK2 activity is regulated by intracellular calcium level, both the temporal and spatial pattern of PYK2 activation, may represent a carbon copy or a replica of the spatial and temporal profile of intracellular calcium concentration. Calcium concentration inside cells is highly localized because of a variety of calcium binding proteins that provide a strong buffer. Moreover, in excitable cells the level of calcium can be regulated by voltage dependent calcium channels that induce large and transient increase in intracellular calcium concentration leading to calcium oscilations and calcium waves. PYK2 may provide a mechanism for rapid and highly localized control of ion channel function, as well as, localized activation of the MAP kinase signaling pathway.

Preliminary inimunolocalization analysis indicates that PYY2 of neurons activity is expressed in hippocampal postsynaptic dendritic spines, suggesting a potential role of this kinase in synaptic plasticity mediated by calcium influx. Potassium channels are

frequent targets for phosphorylation by tyrosine kinases that are activated by neurotransmitters or neuropeptides. Phosphorylation of other voltage gated channels or neurotransmitter receptors provides an important regulatory mechanism for modulation. Thus, PYK2 may represent an important coupling molecule between neuropeptides that activate G-protein coupled receptors or neurotransmitters that stimulate Ca^{+2} influx and downstream signaling events that regulate neuronal plasticity, cell excitability, and synaptic efficacy.

We have demonstrated that PYK2 is rapidly activated in response to a wide variety of extracellular stimuli. These stimuli include activation of an ion channel, stimulation of a G-protein coupled receptor, calcium influx following membrane depolarization as well as phorbol ester stimulation. Although the molecular mechanisms by which these signals induce the activation of PYK2 are not yet known, our results clearly show that elevation of intracellular calcium concentrations is crucial for PYK2 activation. The effect of PMA on PYK2 activation may indicate that PYK2 can be also activated by a PKC dependent pathway. The fact that PYK2 can be activated by an ion-channel, such as the nicotinic acetylcholine receptor, and by intracellular calcium raised the possibility that PYK2 may regulate ion-channel function by tyrosine phosphorylation.

I. Nucleic Acid Encoding A PYK2 Polypeptide.

Included within the scope of this invention are the functional equivalents of the herein-described isolated nucleic acid molecules. The degeneracy of the genetic code permits substitution of certain codons by other codons which specify the same amino acid and hence would give rise to the same protein. The nucleic acid sequence can vary substantially since, with the exception of methionine and tryptophan, the known amino acids can be coded for by more than one codon. Thus, portions or all

of the PYK2 gene could be synthesized to give a nucleic acid sequence significantly different from that shown in SEQ ID NO: 1. The encoded amino acid sequence thereof would, however, be preserved.

5 In addition, the nucleic acid sequence may comprise a nucleotide sequence which results from the addition, deletion or substitution of at least one nucleotide to the 5'-end and/or the 3'-end of the nucleic acid formula shown in SEQ ID NO: 1 or a derivative thereof. Any nucleotide or polynucleotide may be used in
10 this regard, provided that its addition, deletion or substitution does not alter the amino acid sequence of SEQ ID NO:2 which is encoded by the nucleotide sequence. For example, the present invention is intended to include any
15 nucleic acid sequence resulting from the addition of ATG as an initiation codon at the 5'-end of the inventive nucleic acid sequence or its derivative, or from the addition of TTA, TAG or TGA as a termination codon at the 3'-end of the inventive nucleotide sequence or its
20 derivative. Moreover, the nucleic acid molecule of the present invention may, as necessary, have restriction endonuclease recognition sites added to its 5'-end and/or 3'-end.

 Such functional alterations of a given nucleic
25 acid sequence afford an opportunity to promote secretion and/or processing of heterologous proteins encoded by foreign nucleic acid sequences fused thereto. All variations of the nucleotide sequence of the PYK2 genes and fragments thereof permitted by the genetic code are,
30 therefore, included in this invention.

 Further, it is possible to delete codons or to substitute one or more codons by codons other than degenerate codons to produce a structurally modified polypeptide, but one which has substantially the same
35 utility or activity of the polypeptide produced by the unmodified nucleic acid molecule. As recognized in the art, the two polypeptides are functionally equivalent, as

are the two nucleic acid molecules which give rise to their production, even though the differences between the nucleic acid molecules are not related to degeneracy of the genetic code.

5 II. A Nucleic Acid Probe for the Detection of PYK2.

 A nucleic acid probe of the present invention may be used to probe an appropriate chromosomal or cDNA library by usual hybridization methods to obtain another nucleic acid molecule of the present invention. A
10 chromosomal DNA or cDNA library may be prepared from appropriate cells according to recognized methods in the art (cf. Molecular Cloning: A Laboratory Manual, second edition, edited by Sambrook, Fritsch, & Maniatis, Cold Spring Harbor Laboratory, 1989).

15 In the alternative, chemical synthesis is carried out in order to obtain nucleic acid probes having nucleotide sequences which correspond to N-terminal and C-terminal portions of the amino acid sequence of the polypeptide of interest. Thus, the synthesized nucleic
20 acid probes may be used as primers in a polymerase chain reaction (PCR) carried out in accordance with recognized PCR techniques, essentially according to PCR Protocols, A Guide to Methods and Applications, edited by Michael et al., Academic Press, 1990, utilizing the appropriate
25 chromosomal or cDNA library to obtain the fragment of the present invention.

 One skilled in the art can readily design such probes based on the sequence disclosed herein using methods of computer alignment and sequence analysis known
30 in the art (cf. Molecular Cloning: A Laboratory Manual, second edition, edited by Sambrook, Fritsch, & Maniatis, Cold Spring Harbor Laboratory, 1989). The hybridization probes of the present invention can be labeled by standard labeling techniques such as with a radiolabel, enzyme
35 label, fluorescent label, biotin-avidin label,

chemiluminescence, and the like. After hybridization, the probes may be visualized using known methods.

The nucleic acid probes of the present invention include RNA, as well as DNA probes, such probes being
5 generated using techniques known in the art. The nucleic acid probe may be immobilized on a solid support. Examples of such solid supports include, but are not limited to, plastics such as polycarbonate, complex carbohydrates such as agarose and sepharose, and acrylic
10 resins, such as polyacrylamide and latex beads. Techniques for coupling nucleic acid probes to such solid supports are well known in the art.

The test samples suitable for nucleic acid probing methods of the present invention include, for
15 example, cells or nucleic acid extracts of cells, or biological fluids. The sample used in the above-described methods will vary based on the assay format, the detection method and the nature of the tissues, cells or extracts to be assayed. Methods for preparing nucleic acid extracts
20 of cells are well known in the art and can be readily adapted in order to obtain a sample which is compatible with the method utilized.

III. Probe Based Method And Kit For Detecting PYK2.

One method of detecting the presence of PYK2 in
25 a sample comprises a) contacting said sample with the above-described nucleic acid probe, under conditions such that hybridization occurs, and b) detecting the presence of said probe bound to said nucleic acid molecule. One skilled in the art would select the nucleic acid probe
30 according to techniques known in the art as described above. Samples to be tested include but should not be limited to RNA samples of human tissue.

A kit for detecting the presence of PYK2 in a sample comprises at least one container means having
35 disposed therein the above-described nucleic acid probe. The kit may further comprise other containers comprising

one or more of the following: wash reagents and reagents capable of detecting the presence of bound nucleic acid probe. Examples of detection reagents include, but are not limited to radiolabelled probes, enzymatic labeled probes (horse radish peroxidase, alkaline phosphatase),
5 and affinity labeled probes (biotin, avidin, or streptavidin).

In detail, a compartmentalized kit includes any kit in which reagents are contained in separate
10 containers. Such containers include small glass containers, plastic containers or strips of plastic or paper. Such containers allow the efficient transfer of reagents from one compartment to another compartment such that the samples and reagents are not cross-contaminated
15 and the agents or solutions of each container can be added in a quantitative fashion from one compartment to another. Such containers will include a container which will accept the test sample, a container which contains the probe or primers used in the assay, containers which contain wash
20 reagents (such as phosphate buffered saline, Tris-buffers, and the like), and containers which contain the reagents used to detect the hybridized probe, bound antibody, amplified product, or the like. One skilled in the art will readily recognize that the nucleic acid probes
25 described in the present invention can readily be incorporated into one of the established kit formats which are well known in the art.

IV. DNA Constructs Comprising a PYK2 Nucleic Acid Molecule and Cells Containing These Constructs.

30 The present invention also relates to a recombinant DNA molecule comprising, 5' to 3', a promoter effective to initiate transcription in a host cell and the above-described nucleic acid molecules. In addition, the present invention relates to a recombinant DNA molecule
35 comprising a vector and an above-described nucleic acid molecules. The present invention also relates to a

nucleic acid molecule comprising a transcriptional region functional in a cell, a sequence complimentary to an RNA sequence encoding an amino acid sequence corresponding to the above-described polypeptide, and a transcriptional
5 termination region functional in said cell. The above-described molecules may be isolated and/or purified DNA molecules.

The present invention also relates to a cell or organism that contains an above-described nucleic acid
10 molecule. The peptide may be purified from cells which have been altered to express the peptide. A cell is said to be "altered to express a desired peptide" when the cell, through genetic manipulation, is made to produce a protein which it normally does not produce or which the
15 cell normally produces at lower levels. One skilled in the art can readily adapt procedures for introducing and expressing either genomic, cDNA, or synthetic sequences into either eukaryotic or prokaryotic cells.

A nucleic acid molecule, such as DNA, is said to
20 be "capable of expressing" a polypeptide if it contains nucleotide sequences which contain transcriptional and translational regulatory information and such sequences are "operably linked" to nucleotide sequences which encode the polypeptide. An operable linkage is a linkage in
25 which the regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene sequence expression. The precise nature of the regulatory regions needed for gene sequence expression may vary from organism to organism, but shall in general
30 include a promoter region which, in prokaryotes, contains both the promoter (which directs the initiation of RNA transcription) as well as the DNA sequences which, when transcribed into RNA, will signal synthesis initiation. Such regions will normally include those 5'-non-coding
35 sequences involved with initiation of transcription and translation, such as the TATA box, capping sequence, CAAT sequence, and the like.

If desired, the non-coding region 3' to the sequence encoding an PYK2 gene may be obtained by the above-described methods. This region may be retained for its transcriptional termination regulatory sequences, such as termination and polyadenylation. Thus, by retaining the 3'-region naturally contiguous to the DNA sequence encoding an PYK2 gene, the transcriptional termination signals may be provided. Where the transcriptional termination signals are not satisfactorily functional in the expression host cell, then a 3' region functional in the host cell may be substituted.

Two DNA sequences (such as a promoter region sequence and an PYK2 sequence) are said to be operably linked if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region sequence to direct the transcription of an PYK2 gene sequence, or (3) interfere with the ability of the an PYK2 gene sequence to be transcribed by the promoter region sequence. Thus, a promoter region would be operably linked to a DNA sequence if the promoter were capable of effecting transcription of that DNA sequence. Thus, to express an PYK2 gene, transcriptional and translational signals recognized by an appropriate host are necessary.

The present invention encompasses the expression of the PYK2 gene (or a functional derivative thereof) in either prokaryotic or eukaryotic cells. Prokaryotic hosts are, generally, very efficient and convenient for the production of recombinant proteins and are, therefore, one type of preferred expression system for the PYK2 gene. Prokaryotes most frequently are represented by various strains of *E. coli*. However, other microbial strains may also be used, including other bacterial strains.

In prokaryotic systems, plasmid vectors that contain replication sites and control sequences derived from a species compatible with the host may be used.

Examples of suitable plasmid vectors may include pBR322, pUC118, pUC119 and the like; suitable phage or bacteriophage vectors may include γ gt10, γ gt11 and the like; and suitable virus vectors may include pMAM-neo, 5 pKRC and the like. Preferably, the selected vector of the present invention has the capacity to replicate in the selected host cell.

Recognized prokaryotic hosts include bacteria such as *E. coli*, *Bacillus*, *Streptomyces*, *Pseudomonas*, 10 *Salmonella*, *Serratia*, and the like. However, under such conditions, the peptide will not be glycosylated. The prokaryotic host must be compatible with the replicon and control sequences in the expression plasmid.

To express PYK2 (or a functional derivative 15 thereof) in a prokaryotic cell, it is necessary to operably link the PYK2 sequence to a functional prokaryotic promoter. Such promoters may be either constitutive or, more preferably, regulatable (i.e., inducible or derepressible). Examples of constitutive 20 promoters include the int promoter of bacteriophage λ , the bla promoter of the β -lactamase gene sequence of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene sequence of pPR325, and the like. Examples of inducible prokaryotic promoters include the 25 major right and left promoters of bacteriophage λ (P_L and P_R), the trp, recA, lacZ, lacI, and gal promoters of *E. coli*, the α -amylase (Ulmanen et al., J. Bacteriol. 162:176-182(1985)) and the ζ -28-specific promoters of *B. subtilis* (Gilman et al., Gene sequence 32:11-20(1984)), 30 the promoters of the bacteriophages of *Bacillus* (Gryczan, In: The Molecular Biology of the Bacilli, Academic Press, Inc., NY (1982)), and *Streptomyces* promoters (Ward et al., Mol. Gen. Genet. 203:468-478(1986)). Prokaryotic promoters are reviewed by Glick (J. Ind. Microbiot. 1:277- 35 282(1987)); Cenatiempo (Biochimie 68:505-516(1986)); and Gottesman (Ann. Rev. Genet. 18:415-442 (1984)).

Proper expression in a prokaryotic cell also requires the presence of a ribosome binding site upstream of the gene sequence-encoding sequence. Such ribosome binding sites are disclosed, for example, by Gold et al. 5 (Ann. Rev. Microbiol. 35:365-404(1981)). The selection of control sequences, expression vectors, transformation methods, and the like, are dependent on the type of host cell used to express the gene. As used herein, "cell", "cell line", and "cell culture" may be used 10 interchangeably and all such designations include progeny. Thus, the words "transformants" or "transformed cells" include the primary subject cell and cultures derived therefrom, without regard to the number of transfers. It is also understood that all progeny may not be precisely 15 identical in DNA content, due to deliberate or inadvertent mutations. However, as defined, mutant progeny have the same functionality as that of the originally transformed cell.

Host cells which may be used in the expression 20 systems of the present invention are not strictly limited, provided that they are suitable for use in the expression of the PYK2 peptide of interest. Suitable hosts may often include eukaryotic cells. Preferred eukaryotic hosts include, for example, yeast, fungi, insect cells, 25 mammalian cells either in vivo, or in tissue culture. Mammalian cells which may be useful as hosts include HeLa cells, cells of fibroblast origin such as VERO or CHO-K1, or cells of lymphoid origin and their derivatives. Preferred mammalian host cells include SP2/0 and J558L, as 30 well as neuroblastoma cell lines such as IMR 332 which may provide better capacities for correct post-translational processing.

In addition, plant cells are also available as hosts, and control sequences compatible with plant cells 35 are available, such as the cauliflower mosaic virus 35S and 19S, and nopaline synthase promoter and polyadenylation signal sequences. Another preferred host

is an insect cell, for example the *Drosophila* larvae. Using insect cells as hosts, the *Drosophila* alcohol dehydrogenase promoter can be used. Rubin, Science 240:1453-1459(1988). Alternatively, baculovirus vectors
5 can be engineered to express large amounts of PYK2 in insects cells (Jasny, Science 238:1653 (1987); Miller et al., In: Genetic Engineering (1986), Setlow, J.K., et al., eds., Plenum, Vol. 8, pp. 277-297).

Any of a series of yeast gene sequence expression
10 systems can be utilized which incorporate promoter and termination elements from the actively expressed gene sequences coding for glycolytic enzymes are produced in large quantities when yeast are grown in mediums rich in glucose. Known glycolytic gene sequences can also provide
15 very efficient transcriptional control signals. Yeast provides substantial advantages in that it can also carry out post-translational peptide modifications. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number of plasmids which
20 can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian gene sequence products and secretes peptides bearing leader sequences (i.e., pre-peptides). For a mammalian host, several possible vector systems are
25 available for the expression of PYK2.

A wide variety of transcriptional and translational regulatory sequences may be employed, depending upon the nature of the host. The transcriptional and translational regulatory signals may
30 be derived from viral sources, such as adenovirus, bovine papilloma virus, cytomegalovirus, simian virus, or the like, where the regulatory signals are associated with a particular gene sequence which has a high level of expression. Alternatively, promoters from mammalian
35 expression products, such as actin, collagen, myosin, and the like, may be employed. Transcriptional initiation regulatory signals may be selected which allow for

repression or activation, so that expression of the gene sequences can be modulated. Of interest are regulatory signals which are temperature-sensitive so that by varying the temperature, expression can be repressed or initiated, or are subject to chemical (such as metabolite) regulation.

Expression of PYK2 in eukaryotic hosts requires the use of eukaryotic regulatory regions. Such regions will, in general, include a promoter region sufficient to direct the initiation of RNA synthesis. Preferred eukaryotic promoters include, for example, the promoter of the mouse metallothionein I gene sequence (Hamer et al., J. Mol. Appl. Gen. 1:273-288(1982)); the TK promoter of Herpes virus (McKnight, Cell 31:355-365 (1982)); the SV40 early promoter (Benoist et al., Nature (London) 290:304-310(1981)); the yeast gal4 gene sequence promoter (Johnston et al., Proc. Natl. Acad. Sci. (USA) 79:6971-6975(1982); Silver et al., Proc. Natl. Acad. Sci. (USA) 81:5951-5955 (1984)).

Translation of eukaryotic mRNA is initiated at the codon which encodes the first methionine. For this reason, it is preferable to ensure that the linkage between a eukaryotic promoter and a DNA sequence which encodes PYK2 (or a functional derivative thereof) does not contain any intervening codons which are capable of encoding a methionine (i.e., AUG). The presence of such codons results either in a formation of a fusion protein (if the AUG codon is in the same reading frame as the PYK2 coding sequence) or a frame-shift mutation (if the AUG codon is not in the same reading frame as the PYK2 coding sequence).

A PYK2 nucleic acid molecule and an operably linked promoter may be introduced into a recipient prokaryotic or eukaryotic cell either as a nonreplicating DNA (or RNA) molecule, which may either be a linear molecule or, more preferably, a closed covalent circular molecule. Since such molecules are incapable of

autonomous replication, the expression of the gene may occur through the transient expression of the introduced sequence. Alternatively, permanent expression may occur through the integration of the introduced DNA sequence
5 into the host chromosome.

A vector may be employed which is capable of integrating the desired gene sequences into the host cell chromosome. Cells which have stably integrated the introduced DNA into their chromosomes can be selected by
10 also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may provide for prototrophy to an auxotrophic host, biocide resistance, e.g., antibiotics, or heavy metals, such as copper, or the like. The
15 selectable marker gene sequence can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of single chain binding protein mRNA. These
20 elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. cDNA expression vectors incorporating such elements include those described by Okayama, Molec. Cell. Biol. 3:280(1983).

25 The introduced nucleic acid molecule can be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors may be employed for this purpose. Factors of importance in selecting a particular plasmid or
30 viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable
35 to be able to "shuttle" the vector between host cells of different species. Preferred prokaryotic vectors include plasmids such as those capable of replication in E. coli

(such as, for example, pBR322, ColE1, pSC101, pACYC 184, π VX. Such plasmids are, for example, disclosed by Sambrook (cf. Molecular Cloning: A Laboratory Manual, second edition, edited by Sambrook, Fritsch, & Maniatis, Cold Spring Harbor Laboratory, (1989)). Bacillus plasmids include pC194, pC221, pT127, and the like. Such plasmids are disclosed by Gryczan (In: The Molecular Biology of the Bactitli, Academic Press, NY (1982), pp. 307-329). Suitable Streptomyces plasmids include p1J101 (Kendall et al., J. Bacteriol. 169:4177-4183 (1987)), and streptomyces bacteriophages such as ϕ C31 (Chater et al., In: Sixth International Symposium on Actinomycetales Biology, Akademiai Kiado, Budapest, Hungary (1986), pp. 45-54). Pseudomonas plasmids are reviewed by John et al. (Rev. Infect. Dis. 8:693-704(1986)), and Izaki (Jpn. J. Bacteriol. 33:729-742(1978)).

Preferred eukaryotic plasmids include, for example, BPV, vaccinia, SV40, 2-micron circle, and the like, or their derivatives. Such plasmids are well known in the art (Botstein et al., Miami Wntr. Symp. 19:265-274(1982); Broach, In: The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p. 445-470 (1981); Broach, Cell 28:203-204 (1982); Bollon et al., J. Clin. Hematol. Oncol. 10:39-48 (1980); Maniatis, In: Cell Biology: A Comprehensive Treatise, Vol. 3, Gene Sequence Expression, Academic Press, NY, pp. 563-608(1980).

Once the vector or nucleic acid molecule containing the construct(s) has been prepared for expression, the DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means, i.e., transformation, transfection, conjugation, protoplast fusion, electroporation, particle gun technology, calcium phosphate-precipitation, direct microinjection, and the like. After the introduction of the vector, recipient cells are grown in a selective medium, which selects for the growth of vector-containing

cells. Expression of the cloned gene molecule(s) results in the production of PYK2 or fragments thereof. This can take place in the transformed cells as such, or following the induction of these cells to differentiate (for
5 example, by administration of bromodeoxyuracil to neuroblastoma cells or the like). A variety of incubation conditions can be used to form the peptide of the present invention. The most preferred conditions are those which mimic physiological conditions.

10 V. Purified PYK2 Polypeptides.

A variety of methodologies known in the art can be utilized to obtain the peptide of the present invention. The peptide may be purified from tissues or cells which naturally produce the peptide. Alternatively,
15 the above-described isolated nucleic acid fragments could be used to express the PYK2 protein in any organism. The samples of the present invention include cells, protein extracts or membrane extracts of cells, or biological fluids. The sample will vary based on the
20 assay format, the detection method and the nature of the tissues, cells or extracts used as the sample.

Any eukaryotic organism can be used as a source for the peptide of the invention, as long as the source organism naturally contains such a peptide. As used
25 herein, "source organism" refers to the original organism from which the amino acid sequence of the subunit is derived, regardless of the organism the subunit is expressed in and ultimately isolated from.

One skilled in the art can readily follow known
30 methods for isolating proteins in order to obtain the peptide free of natural contaminants. These include, but are not limited to: size-exclusion chromatography, HPLC, ion-exchange chromatography, and immuno-affinity chromatography.

VI. PYK2 Antibody And Hybridoma.

The present invention relates to an antibody having binding affinity to a PYK2 polypeptide. The polypeptide may have the amino acid sequence set forth in SEQ ID NO:2, or mutant or species variation thereof, or at
5 least 9 contiguous amino acids thereof (preferably, at least 10, 15, 20, or 30 contiguous amino acids thereof).

The present invention also relates to an antibody having specific binding affinity to an PYK2 polypeptide.
10 Such an antibody may be isolated by comparing its binding affinity to a PYK2 polypeptide with its binding affinity to another polypeptide. Those which bind selectively to PYK2 would be chosen for use in methods requiring a distinction between PYK2 and other polypeptides. Such
15 methods could include, but should not be limited to, the analysis of altered PYK2 expression in tissue containing other polypeptides such as FAK.

The PYK2 proteins of the present invention can be used in a variety of procedures and methods, such as for
20 the generation of antibodies, for use in identifying pharmaceutical compositions, and for studying DNA/protein interaction.

The PYK2 peptide of the present invention can be used to produce antibodies or hybridomas. One skilled in
25 the art will recognize that if an antibody is desired, such a peptide would be generated as described herein and used as an immunogen. The antibodies of the present invention include monoclonal and polyclonal antibodies, as well fragments of these antibodies, and humanized forms.
30 Humanized forms of the antibodies of the present invention may be generated using one of the procedures known in the art such as chimerization or CDR grafting. The present invention also relates to a hybridoma which produces the above-described monoclonal antibody, or binding fragment
35 thereof. A hybridoma is an immortalized cell line which is capable of secreting a specific monoclonal antibody.

In general, techniques for preparing monoclonal antibodies and hybridomas are well known in the art (Campbell, "Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology,"
5 Elsevier Science Publishers, Amsterdam, The Netherlands (1984); St. Groth et al., J. Immunol. Methods 35:1-21(1980)). Any animal (mouse, rabbit, and the like) which is known to produce antibodies can be immunized with the selected polypeptide. Methods for immunization are
10 well known in the art. Such methods include subcutaneous or intraperitoneal injection of the polypeptide. One skilled in the art will recognize that the amount of polypeptide used for immunization will vary based on the animal which is immunized, the antigenicity of the
15 polypeptide and the site of injection.

The polypeptide may be modified or administered in an adjuvant in order to increase the peptide antigenicity. Methods of increasing the antigenicity of a polypeptide are well known in the art. Such procedures
20 include coupling the antigen with a heterologous protein (such as globulin or β -galactosidase) or through the inclusion of an adjuvant during immunization.

For monoclonal antibodies, spleen cells from the immunized animals are removed, fused with myeloma cells, such as SP2/0-Ag14 myeloma cells, and allowed to become
25 monoclonal antibody producing hybridoma cells. Any one of a number of methods well known in the art can be used to identify the hybridoma cell which produces an antibody with the desired characteristics. These include screening
30 the hybridomas with an ELISA assay, western blot analysis, or radioimmunoassay (Lutz et al., Exp. Cell Res. 175:109-124(1988)). Hybridomas secreting the desired antibodies are cloned and the class and subclass is determined using procedures known in the art (Campbell, Monoclonal Antibody
35 Technology: Laboratory Techniques in Biochemistry and Molecular Biology, supra (1984)).

For polyclonal antibodies, antibody containing antisera is isolated from the immunized animal and is screened for the presence of antibodies with the desired specificity using one of the above-described procedures.

5 The above-described antibodies may be detectably labeled. Antibodies can be detectably labeled through the use of radioisotopes, affinity labels (such as biotin, avidin, and the like), enzymatic labels (such as horse radish peroxidase, alkaline phosphatase, and the like)
10 fluorescent labels (such as FITC or rhodamine, and the like), paramagnetic atoms, and the like. Procedures for accomplishing such labeling are well-known in the art, for example, see (Stemberger et al., J. Histochem. Cytochem. 18:315(1970); Bayer et al., Meth. Enzym. 62:308(1979);
15 Engval et al., Immunot. 109:129(1972); Goding, J. Immunol. Meth. 13:215(1976)). The labeled antibodies of the present invention can be used for in vitro, in vivo, and in situ assays to identify cells or tissues which express a specific peptide.

20 The above-described antibodies may also be immobilized on a solid support. Examples of such solid supports include plastics such as polycarbonate, complex carbohydrates such as agarose and sepharose, acrylic resins and such as polyacrylamide and latex beads.
25 Techniques for coupling antibodies to such solid supports are well known in the art (Weir et al., "Handbook of Experimental Immunology" 4th Ed., Blackwell Scientific Publications, Oxford, England, Chapter 10(1986); Jacoby et al., Meth. Enzym. 34 Academic Press, N.Y. (1974)). The
30 immobilized antibodies of the present invention can be used for in vitro, in vivo, and in situ assays as well as in immunochromatography.

Furthermore, one skilled in the art can readily adapt currently available procedures, as well as the
35 techniques, methods and kits disclosed above with regard to antibodies, to generate peptides capable of binding to a specific peptide sequence in order to generate

rationally designed antipeptide peptides, for example see Hurby et al., "Application of Synthetic Peptides: Antisense Peptides", In Synthetic Peptides, A User's Guide, W.H. Freeman, NY, pp. 289-307(1992), and Kaspczak
5 et al., Biochemistry 28:9230-8(1989).

Anti-peptide peptides can be generated by replacing the basic amino acid residues found in the PYK2 peptide sequence with acidic residues, while maintaining hydrophobic and uncharged polar groups. For example,
10 lysine, arginine, and/or histidine residues are replaced with aspartic acid or glutamic acid and glutamic acid residues are replaced by lysine, arginine or histidine.

VII. An Antibody Based Method And Kit For Detecting PYK2.

15 The present invention encompasses a method of detecting an PYK2 polypeptide in a sample, comprising: a) contacting the sample with an above-described antibody, under conditions such that immunocomplexes form, and b) detecting the presence of said antibody bound to the
20 polypeptide. In detail, the methods comprise incubating a test sample with one or more of the antibodies of the present invention and assaying whether the antibody binds to the test sample. Altered levels of PYK2 in a sample as compared to normal levels may indicate muscular disease.

25 Conditions for incubating an antibody with a test sample vary. Incubation conditions depend on the format employed in the assay, the detection methods employed, and the type and nature of the antibody used in the assay. One skilled in the art will recognize that any one of the
30 commonly available immunological assay formats (such as radioimmunoassays, enzyme-linked immunosorbent assays, diffusion based Ouchterlony, or rocket immunofluorescent assays) can readily be adapted to employ the antibodies of the present invention. Examples of such assays can be
35 found in Chard, "An Introduction to Radioimmunoassay and Related Techniques" Elsevier Science Publishers,

Amsterdam, The Netherlands (1986); Bullock et al., "Techniques in Immunocytochemistry," Academic Press, Orlando, FL Vol. 1(1982), Vol. 2 (1983), Vol. 3 (1985); Tijssen, "Practice and Theory of Enzyme Immunoassays: 5 Laboratory Techniques in Biochemistry and Molecular Biology," Elsevier Science Publishers, Amsterdam, The Netherlands (1985).

The immunological assay test samples of the present invention include cells, protein or membrane 10 extracts of cells, or biological fluids such as blood, serum, plasma, or urine. The test sample used in the above-described method will vary based on the assay format, nature of the detection method and the tissues, cells or extracts used as the sample to be assayed. 15 Methods for preparing protein extracts or membrane extracts of cells are well known in the art and can be readily be adapted in order to obtain a sample which is capable with the system utilized.

A kit contains all the necessary reagents to 20 carry out the previously described methods of detection. The kit may comprise: i) a first container means containing an above-described antibody, and ii) second container means containing a conjugate comprising a binding partner of the antibody and a label. In another 25 preferred embodiment, the kit further comprises one or more other containers comprising one or more of the following: wash reagents and reagents capable of detecting the presence of bound antibodies.

Examples of detection reagents include, but are 30 not limited to, labeled secondary antibodies, or in the alternative, if the primary antibody is labeled, the chromophoric, enzymatic, or antibody binding reagents which are capable of reacting with the labeled antibody. The compartmentalized kit may be as described above for 35 nucleic acid probe kits. One skilled in the art will readily recognize that the antibodies described in the present invention can readily be incorporated into one of

the established kit formats which are well known in the art.

VIII. Isolation of Compounds Which Interact With PYK2.

5 The present invention also relates to a method of detecting a compound capable of binding to a PYK2 polypeptide comprising incubating the compound with PYK2 and detecting the presence of the compound bound to PYK2. The compound may be present within a complex mixture, for
10 example, serum, body fluid, or cell extracts.

The present invention also relates to a method of detecting an agonist or antagonist of PYK2 activity comprising incubating cells that produce PYK2 in the presence of a compound and detecting changes in the level
15 of PYK2 activity. The compounds thus identified would produce a change in activity indicative of the presence of the compound. The compound may be present within a complex mixture, for example, serum, body fluid, or cell extracts. Once the compound is identified it can be
20 isolated using techniques well known in the art.

The present invention also encompasses a method of agonizing (stimulating) or antagonizing PYK2 associated activity in a mammal comprising administering to said mammal an agonist or antagonist to PYK2 in an amount
25 sufficient to effect said agonism or antagonism. A method of treating diabetes mellitus, skeletal muscle diseases, Alzheimer's disease, or peripheral neuropathies in a mammal with an agonist or antagonist of PYK2 activity comprising administering the agonist or antagonist to a
30 mammal in an amount sufficient to agonize or antagonize PYK2 associated functions is also encompassed in the present application.

IX. Transgenic Animals.

A variety of methods are available for the
35 production of transgenic animals associated with this

invention. DNA can be injected into the pronucleus of a fertilized egg before fusion of the male and female pronuclei, or injected into the nucleus of an embryonic cell (e.g., the nucleus of a two-cell embryo) following
5 the initiation of cell division (Brinster et al., Proc. Nat. Acad. Sci. USA 82: 4438-4442 (1985)). Embryos can be infected with viruses, especially retroviruses, modified to carry inorganic-ion receptor nucleotide sequences of the invention.

10 Pluripotent stem cells derived from the inner cell mass of the embryo and stabilized in culture can be manipulated in culture to incorporate nucleotide sequences of the invention. A transgenic animal can be produced from such cells through implantation into a blastocyst
15 that is implanted into a foster mother and allowed to come to term. Animals suitable for transgenic experiments can be obtained from standard commercial sources such as Charles River (Wilmington, MA), Taconic (Germantown, NY), Harlan Sprague Dawley (Indianapolis, IN), etc.

20 The procedures for manipulation of the rodent embryo and for microinjection of DNA into the pronucleus of the zygote are well known to those of ordinary skill in the art (Hogan et al., supra). Microinjection procedures for fish, amphibian eggs and birds are detailed in
25 Houdebine and Chourrout, Experientia 47: 897-905 (1991). Other procedures for introduction of DNA into tissues of animals are described in U.S. Patent No., 4,945,050 (Sandford et al., July 30, 1990).

By way of example only, to prepare a transgenic
30 mouse, female mice are induced to superovulate. Females are placed with males, and the mated females are sacrificed by CO₂ asphyxiation or cervical dislocation and embryos are recovered from excised oviducts. Surrounding cumulus cells are removed. Pronuclear embryos are then
35 washed and stored until the time of injection. Randomly cycling adult female mice are paired with vasectomized males. Recipient females are mated at the same time as

donor females. Embryos then are transferred surgically. The procedure for generating transgenic rats is similar to that of mice. See Hammer *et al.*, Cell 63:1099-1112 (1990).

5 Methods for the culturing of embryonic stem (ES) cells and the subsequent production of transgenic animals by the introduction of DNA into ES cells using methods such as electroporation, calcium phosphate/DNA precipitation and direct injection also are well known to
10 those of ordinary skill in the art. See, for example, Teratocarcinomas and Embryonic Stem Cells, A Practical Approach, E.J. Robertson, ed., IRL Press (1987).

 In cases involving random gene integration, a clone containing the sequence(s) of the invention is co-
15 transfected with a gene encoding resistance. Alternatively, the gene encoding neomycin resistance is physically linked to the sequence(s) of the invention. Transfection and isolation of desired clones are carried out by any one of several methods well known to those of
20 ordinary skill in the art (E.J. Robertson, *supra*).

 DNA molecules introduced into ES cells can also be integrated into the chromosome through the process of homologous recombination. Capecchi, Science 244: 1288-1292 (1989). Methods for positive selection of the
25 recombination event (*i.e.*, neo resistance) and dual positive-negative selection (*i.e.*, neo resistance and gancyclovir resistance) and the subsequent identification of the desired clones by PCR have been described by Capecchi, *supra* and Joyner *et al.*, Nature 338: 153-156
30 (1989), the teachings of which are incorporated herein. The final phase of the procedure is to inject targeted ES cells into blastocysts and to transfer the blastocysts into pseudopregnant females. The resulting chimeric animals are bred and the offspring are analyzed by
35 Southern blotting to identify individuals that carry the transgene. Procedures for the production of non-rodent mammals and other animals have been discussed by others.

See Houdebine and Chourrout, supra; Pursel et al., Science 244:1281-1288 (1989); and Simms et al., Bio/Technology 6:179-183 (1988).

X. Compositions

5 The present invention relates to removing or reducing an abnormality in a signal transduction pathway, wherein the signal transduction pathway contains a PYK2 polypeptide. The present invention also relates to compositions and methods for the treatment of disorders
10 which involve modulating the activity and/or level of individual components, and relates to methods for the identification of agents for such treatments. Additionally, the present invention relates to methods and compositions for prognostic evaluation of such disorders.

15 Described herein are compositions and methods for the prevention, prognostic evaluation, and treatment of disorders described herein, preferably cell proliferative disorders and hematopoietic cell disorders, in which a PYK2 polypeptide may be involved.

20 First, methods and compositions for the treatment of such disorders are described. Such methods and compositions may include, but are not limited to the agents capable of decreasing or inhibiting the interaction between a PYK2 polypeptide and a PYK2 polypeptide binding
25 partner and agents capable of inhibiting or decreasing the activity of such complexes, agents capable of modulating the activity and/or level of individual components of the proteins, and the use and administration of such agents. Agents capable of modulating the activity and/or level of
30 interaction between a PYK2 polypeptide and a PYK2 polypeptide binding partner include those agents that inhibit or decrease the dephosphorylating activity of tyrosine phosphatases.

 Second, methods are described for the identification of such agents. These methods may include, for
35 example, assays to identify agents capable of disrupting

or inhibiting or promoting the interaction between components of the complexes (e.g., PYK2:NBP complexes), and may also include paradigms and strategies for the rational design of drugs capable of disruption and/or inhibition
5 and/or promotion of such complexes.

The complexes involved in the invention include a PYK2 polypeptide and a NBP or derivatives thereof, as described below. Under standard physiological conditions, the components of such complexes are capable of forming
10 stable, non-covalent attachments with one or more of the other complex components. Methods for the purification and production of such protein complexes, and of cells that exhibit such complexes are described below.

XI. Disruption of Protein Complexes

15 Disruption of complexes (e.g., PYK2:NBP complexes), for example by decreasing or inhibiting the interactions between component members of such a complex may have differing modulatory effects on the event involved, depending on the individual protein complex.
20 "Disruption", as used here, is meant to refer not only to a physical separation of protein complex components, but also refers to a perturbation of the activity of the complexes, regardless of whether or not such complexes remain able, physically, to form. "Activity", as used
25 here, refers to the function of the protein complex in the signal transduction cascade of the cell in which such a complex is formed, i.e., refers to the function of the complex in effecting or inhibiting a transduction of an extracellular signal into a cell. For example, the effect
30 of complex disruption may augment, reduce, or block a signal normally transduced into the cell. Likewise, depending on the disorder involved, either augmentation, reduction, or blockage of a signal normally transduced into the cell will be desirable for the treatment of the
35 disorder.

A disorder involving a complex may, for example, develop because the presence of such a complex brings about the aberrant inhibition of a normal signal transduction event. In such a case, the disruption of the complex would allow the restoration of the usual signal transduction event. Further, an aberrant complex may bring about an altered subcellular adapter protein localization, which may result in, for example, dysfunctional cellular events. An inhibition of the complex in this case would allow for restoration or maintenance of a normal cellular architecture. Still further, an agent or agents that cause(s) disruption of the complex may bring about the disruption of the interactions among other potential components of a complex.

Nucleotide sequences encoding peptide agents which are to be utilized intracellularly may be expressed in the cells of interest, using techniques which are well known to those of ordinary skill in the art. For example, expression vectors derived from viruses such as retroviruses, vaccinia virus, adenoviruses, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery and expression of such nucleotide sequences into the targeted cell population. Methods for the construction of such vectors are well known. See, for example, the techniques described in Maniatis et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and in Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y., 1989. Complex-binding domains can be identified using, for example, techniques such as those described in Rotin et al. (Rotin et al., *EMBO J.* 11:559-567, 1992), Songyang et al. (Songyang et al., *Cell* 72:767-778, 1993), Felder et al., *Mol. Cell. Biol.* 13:1449-1455, 1993), Fantl et al. (*Cell* 69:413-422, 1992), and Domchek et al. (*Biochemistry* 31:9865-9870, 1992).

Alternatively, antibodies capable of interfering with complex formation may be produced as described below and administered for the treatment of disorders involving a component capable of forming a complex with another protein. Alternatively, nucleotide sequences encoding single-chain antibodies may be expressed within the target cell population by utilizing, for example, techniques such as those described in Marasco et al. (Marasco et al., *Proc. Natl. Acad. Sci. USA* 90:7889-7893, 1993). Agents which act intracellularly to interfere with the formation and/or activity of the protein complexes of the invention may also be small organic or inorganic compounds. A method for identifying these and other intracellular agents is described below.

XII. Antibodies to Complexes

Described herein are methods for the production of antibodies which are capable of specifically recognizing a complex or an epitope thereof, or of specifically recognizing an epitope on either of the components of the complex, especially those epitopes which would not be recognized by the antibody when the component is present separate and apart from the complex. Such antibodies may include, but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab'), fragments, fragments produced by a FAb expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. Such antibodies may be used, for example, in the detection of a complex in a biological sample, or, alternatively, as a method for the inhibition of a complex formation, thus inhibiting the development of a disorder.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen, such as a complex, or an antigenic functional derivative thereof. For the pro-

duction of polyclonal antibodies, various host animals may be immunized by injection with the complex including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

A monoclonal antibody, which is a substantially homogeneous population of antibodies to a particular antigen, may be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to the hybridoma technique of Kohler and Milstein (Nature 256:495-497, 1975) and U.S. Patent No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., Immunology Today 4:72, 1983; Cole et al., Proc. Natl. Acad. Sci. USA 80:2026-2030, 1983), and the EBV-hybridoma technique (Cole et al., Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., 1985, pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated in vitro or in vivo. Production of high titers of mAbs in vivo makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., Proc. Natl. Acad. Sci., 81:6851-6855, 1984; Neuberger et al., Nature, 312:604-608, 1984; Takeda et al., Nature, 314:452-454, 1985) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is

a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region.

5 Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; Bird, *Science* 242:423-426, 1988; Huston et al., *Proc. Natl. Acad. Sci. USA* 85:5879-5883, 1988; and Ward et al., *Nature* 334:544-546, 1989) can be adapted to produce
10 complex-specific single chain antibodies. Single chain antibodies are formed by linking the heavy and light chain fragment of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

 Antibody fragments which contain specific binding
15 sites of a complex may be generated by known techniques. For example, such fragments include but are not limited to: the F(ab'), fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges
20 of the F(ab'), fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, *Science*, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity to the PTK/adaptor complex.

25 One or more components of a protein complex may be present at a higher than normal cellular level (i.e., higher than the concentration known to usually be present in the cell type exhibiting the protein complex of interest) and/or may exhibit an abnormally increased level of
30 cellular activity (i.e., greater than the activity known to usually be present in the cell type exhibiting the protein complex of interest).

 For example, the gene encoding a protein complex component may begin to be overexpressed, or may be amplified (i.e., its gene copy number may be increased) in
35 certain cells, leading to an increased number of component molecules within these cells. Additionally, a gene

encoding a protein complex component may begin to express a modified protein product that exhibits a greater than normal level of activity. "Activity", here, refers to the normal cellular function of the component, either enzymatic or structural whose function may include, for example, bringing two or more cellular molecules into the appropriate proximity.

Such an increase in the cellular level and/or activity of a protein complex may lead to the development of a disorder. Treatment of such disorders may, therefore, be effectuated by the administration of agents which decrease the cellular level and/or the activity of the overexpressed and/or overactive protein complex component. Techniques for decreasing the cellular level and/or the activity of one or more of the protein complex components of interest may include, but are not limited to antisense or ribozyme approaches, and/or gene therapy approaches, each of which is well known to those of skill in the art.

XIII. Antisense and Ribozyme Approaches

Included in the scope of the invention are oligoribonucleotides, including antisense RNA and DNA molecules and ribozymes that function to inhibit translation of one or more components of a protein complex. Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. With respect to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g., between -10 and +10 regions of the relevant nucleotide sequence, are preferred.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific interaction of the ribozyme molecule to complementary target RNA, followed by a endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead or other motif ribozyme molecules that specifically and efficiently

catalyze endonucleolytic cleavage of RNA sequences encoding protein complex components.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning
5 the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for
10 predicted structural features, such as secondary structure, that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease
15 protection assays. See, Draper PCT WO 93/23569.

Both anti-sense RNA and DNA molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of RNA molecules. See, Draper, *id.* hereby incorporated by reference herein.
20 These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences
25 encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can
30 be introduced stably into cell lines.

Various modifications to the DNA molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are
35 not limited to the addition of flanking sequences of ribo- or deoxy- nucleotides to the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl

rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

XIV. Gene Therapy

PYK2 or its genetic sequences will also be useful
5 in gene therapy (reviewed in Miller, *Nature* 357:455-460,
(1992). Miller states that advances have resulted in
practical approaches to human gene therapy that have
demonstrated positive initial results. An *in vivo* model
of gene therapy for human severe combined immunodeficiency
10 is described in Ferrari, et al., *Science* 251:1363-1366,
(1991). The basic science of gene therapy is described in
Mulligan, *Science* 260:926-931, (1993).

In one preferred embodiment, an expression vector
containing the PYK2 coding sequence is inserted into
15 cells, the cells are grown *in vitro* and then infused in
large numbers into patients. In another preferred embodi-
ment, a DNA segment containing a promoter of choice (for
example a strong promoter) is transferred into cells
containing an endogenous PYK2 in such a manner that the
20 promoter segment enhances expression of the endogenous
PYK2 gene (for example, the promoter segment is
transferred to the cell such that it becomes directly
linked to the endogenous PYK2 gene).

The gene therapy may involve the use of an
25 adenovirus containing PYK2 cDNA targeted to a tumor,
systemic PYK2 increase by implantation of engineered
cells, injection with PYK2 virus, or injection of naked
PYK2 DNA into appropriate tissues.

Target cell populations (e.g., hematopoietic or
30 nerve cells) may be modified by introducing altered forms
of PYK2 in order to modulate the activity of such cells.
For example, by reducing or inhibiting an a nerve cell
within target cells, an abnormal response leading to a
condition may be decreased, inhibited, or reversed.
35 Deletion or missense mutants of PYK2, that retain the
ability to interact with other components of the nervous

system but cannot participate in normal function may be used to inhibit an abnormal, deleterious response.

Expression vectors derived from viruses such as retroviruses, vaccinia virus, adenovirus, adeno-associated virus, herpes viruses, several RNA viruses, or bovine papilloma virus, may be used for delivery of nucleotide sequences (e.g., cDNA) encoding recombinant PYK2 protein into the targeted cell population (e.g., tumor cells). Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors containing coding sequences. See, for example, the techniques described in Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. (1989), and in Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y. (1989). Alternatively, recombinant nucleic acid molecules encoding protein sequences can be used as naked DNA or in reconstituted system e.g., liposomes or other lipid systems for delivery to target cells (See e.g., Felgner et al., Nature 337:387-8, 1989). Several other methods for the direct transfer of plasmid DNA into cells exist for use in human gene therapy and involve targeting the DNA to receptors on cells by complexing the plasmid DNA to proteins. See, Miller, *supra*.

In its simplest form, gene transfer can be performed by simply injecting minute amounts of DNA into the nucleus of a cell, through a process of microinjection. Capecchi MR, Cell 22:479-88 (1980). Once recombinant genes are introduced into a cell, they can be recognized by the cells normal mechanisms for transcription and translation, and a gene product will be expressed. Other methods have also been attempted for introducing DNA into larger numbers of cells. These methods include: transfection, wherein DNA is precipitated with CaPO₄ and taken into cells by pinocytosis (Chen C. and Okayama H, Mol. Cell Biol. 7:2745-52

(1987)); electroporation, wherein cells are exposed to large voltage pulses to introduce holes into the membrane (Chu G. et al., Nucleic Acids Res., 15:1311-26 (1987)); lipofection/liposome fusion, wherein DNA is packaged into lipophilic vesicles which fuse with a target cell (Felgner PL., et al., Proc. Natl. Acad. Sci. USA. 84:7413-7 (1987)); and particle bombardment using DNA bound to small projectiles (Yang NS. et al., Proc. Natl. Acad. Sci. 87:9568-72 (1990)). Another method for introducing DNA into cells is to couple the DNA to chemically modified proteins.

It has also been shown that adenovirus proteins are capable of destabilizing endosomes and enhancing the uptake of DNA into cells. The admixture of adenovirus to solutions containing DNA complexes, or the binding of DNA to polylysine covalently attached to adenovirus using protein crosslinking agents substantially improves the uptake and expression of the recombinant gene. Curiel DT et al., Am. J. Respir. Cell. Mol. Biol., 6:247-52 (1992).

As used herein "gene transfer" means the process of introducing a foreign nucleic acid molecule into a cell. Gene transfer is commonly performed to enable the expression of a particular product encoded by the gene. The product may include a protein, polypeptide, anti-sense DNA or RNA, or enzymatically active RNA. Gene transfer can be performed in cultured cells or by direct administration into animals. Generally gene transfer involves the process of nucleic acid contact with a target cell by non-specific or receptor mediated interactions, uptake of nucleic acid into the cell through the membrane or by endocytosis, and release of nucleic acid into the cytoplasm from the plasma membrane or endosome. Expression may require, in addition, movement of the nucleic acid into the nucleus of the cell and binding to appropriate nuclear factors for transcription.

As used herein "gene therapy" is a form of gene transfer and is included within the definition of gene

transfer as used herein and specifically refers to gene transfer to express a therapeutic product from a cell in vivo or in vitro. Gene transfer can be performed ex vivo on cells which are then transplanted into a patient, or
5 can be performed by direct administration of the nucleic acid or nucleic acid-protein complex into the patient.

In another preferred embodiment, a vector having nucleic acid sequences encoding PYK2 is provided in which the nucleic acid sequence is expressed only in specific
10 tissue. Methods of achieving tissue-specific gene expression as set forth in International Publication No. WO 93/09236, filed November 3, 1992 and published May 13, 1993.

In all of the preceding vectors set forth above,
15 a further aspect of the invention is that the nucleic acid sequence contained in the vector may include additions, deletions or modifications to some or all of the sequence of the nucleic acid, as defined above.

In another preferred embodiment, a method of gene
20 replacement is set forth. "Gene replacement" as used herein means supplying a nucleic acid sequence which is capable of being expressed in vivo in an animal and thereby providing or augmenting the function of an endogenous gene which is missing or defective in the
25 animal.

XV. Pharmaceutical Formulations and Modes of Administration

The particular compound, antibody, antisense or ribozyme molecule that affects the protein complexes and
30 the disorder of interest can be administered to a patient either by themselves, or in pharmaceutical compositions where it is mixed with suitable carriers or excipient(s). In treating a patient exhibiting a disorder of interest, a therapeutically effective amount of a agent or agents
35 such as these is administered. A therapeutically effective dose refers to that amount of the compound that

results in amelioration of symptoms or a prolongation of survival in a patient.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50} . Compounds which exhibit large therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized.

For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} as determined in cell culture (i.e., the concentration of the test compound which achieves a half-maximal disruption of the protein complex, or a half-maximal inhibition of the cellular level and/or activity of a complex component). Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by HPLC.

The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g. Fingl et al., in The Pharmacological Basis of Therapeutics, 1975, Ch. 1 p. 1). It should be noted that the attending physician would know how to and when to terminate, interrupt, or adjust administration due to toxicity, or to organ dys-

functions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administered dose in the management
5 of the oncogenic disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose
10 frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above may be used in veterinary medicine.

Depending on the specific conditions being
15 treated, such agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, PA (1990). Suitable routes may include oral, rectal,
20 transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections,
25 just to name a few.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For
30 such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

Use of pharmaceutically acceptable carriers to formulate the compounds herein disclosed for the practice
35 of the invention into dosages suitable for systemic administration is within the scope of the invention. With proper choice of carrier and suitable manufacturing

practice, the compositions of the present invention, in particular, those formulated as solutions, may be administered parenterally, such as by intravenous injection. The compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the invention to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated.

Agents intended to be administered intracellularly may be administered using techniques well known to those of ordinary skill in the art. For example, such agents may be encapsulated into liposomes, then administered as described above. Liposomes are spherical lipid bilayers with aqueous interiors. All molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external microenvironment and, because liposomes fuse with cell membranes, are efficiently delivered into the cell cytoplasm. Additionally, due to their hydrophobicity, small organic molecules may be directly administered intracellularly.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions. The pharmaceutical compositions

of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levitating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc,

polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings
5 for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules
10 can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds
15 may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.

Some methods of delivery that may be used include:

- 20 a. encapsulation in liposomes,
- b. transduction by retroviral vectors,
- c. localization to nuclear compartment utilizing nuclear targeting site found on most nuclear proteins,
- 25 d. transfection of cells ex vivo with subsequent reimplantation or administration of the transfected cells,
- e. a DNA transporter system.

A PYK2 nucleic acid sequence may be administered
30 utilizing an ex vivo approach whereby cells are removed from an animal, transduced with the PYK2 nucleic acid sequence and reimplanted into the animal. The liver can be accessed by an ex vivo approach by removing hepatocytes from an animal, transducing the hepatocytes in vitro with
35 the PYK2 nucleic acid sequence and reimplanting them into the animal (e.g., as described for rabbits by Chowdhury et al, Science 254: 1802-1805, 1991, or in humans by Wilson,

Hum. Gene Ther. 3: 179-222, 1992) incorporated herein by reference.

Many nonviral techniques for the delivery of a PYK2 nucleic acid sequence into a cell can be used, including direct naked DNA uptake (e.g., Wolff et al., Science 247: 1465-1468, 1990), receptor-mediated DNA uptake, e.g., using DNA coupled to asialoglycoprotein which is taken up by the asialoglycoprotein receptor in the liver (Wu and Wu, J. Biol. Chem. 262: 4429-4432, 1987; Wu et al., J. Biol. Chem. 266: 14338-14342, 1991), and liposome-mediated delivery (e.g., Kaneda et al., Expt. Cell Res. 173: 56-69, 1987; Kaneda et al., Science 243: 375-378, 1989; Zhu et al., Science 261: 209-211, 1993). Many of these physical methods can be combined with one another and with viral techniques; enhancement of receptor-mediated DNA uptake can be effected, for example, by combining its use with adenovirus (Curiel et al., Proc. Natl. Acad. Sci. USA 88: 8850-8854, 1991; Cristiano et al., Proc. Natl. Acad. Sci. USA 90: 2122-2126, 1993).

The PYK2 or nucleic acid encoding PYK2 may also be administered via an implanted device that provides a support for growing cells. Thus, the cells may remain in the implanted device and still provide the useful and therapeutic agents of the present invention.

XVI. Identification of Agents

The complexes, components of such complexes, functional equivalents thereof, and/or cell lines that express such components and exhibit such protein complexes may be used to screen for additional compounds, antibodies, or other molecules capable of modulating the signal transduction event such complexes are involved in. Methods for purifying and/or producing such complexes, components of the complexes, functional equivalents thereof, and/or cell lines are described herein. The compounds, antibodies, or other molecules identified may, for example, act to disrupt the protein complexes of the

invention (i.e., decrease or inhibit interactions between component members of the complexes, thereby causing physical separation of the components, and/or perturbing the activity of the complexes) or may lower the cellular level and/or decrease the activity of one or more of the components of such complexes.

Such compounds may include, but are not limited to, peptides made of D- and/or L-configuration amino acids (in, for example, the form of random peptide libraries; see Lam et al., *Nature* 354:82-84, 1991), phosphopeptides (in, for example, the form of random or partially degenerate, directed phosphopeptide libraries, see Songyang et al., *Cell* 767-778, 1993), antibodies, and small organic or inorganic molecules. Synthetic compounds, natural products, and other sources of potentially biologically active materials may be screened in a variety of ways, as described herein. The compounds, antibodies, or other molecules identified may be used as oncogenic disorder treatments, as described herein.

Compounds that bind to individual components, or functional portions of the individual components of the complexes (and may additionally be capable of disrupting complex formation) may be identified.

One such method included within the scope of the invention is a method for identifying an agent to be tested for an ability to modulate a signal transduction pathway disorder. The method involves exposing at least one agent to a protein comprising a functional portion of a member of the protein complex for a time sufficient to allow binding of the agent to the functional portion of the member; removing non-bound agents; and determining the presence of the compound bound to the functional portion of the member of the protein complex, thereby identifying an agent to be tested for an ability to modulate a disorder involving a polypeptide complex.

By "signal transduction disorder" is meant any disease or condition associated with an abnormality in a

signal transduction pathway. The protein complex referred to below is a physical association of dynamin and a PYK2 polypeptide. The level of interaction between the two components of the complex may be abnormal and thus cause
5 the abnormality in the signal transduction pathway. Alternatively, the level of interaction between the complex components may be normal, but affecting that interaction may effectively treat a signal transduction pathway disorder.

10 The term "protein" refers to a compound formed of 5-50 or more amino acids joined together by peptide bonds. An "amino acid" is a subunit that is polymerized to form proteins and there are twenty amino acids that are universally found in proteins. The general formula for an
15 amino acid is $H_2N-CHR-COOH$, in which the R group can be anything from a hydrogen atom (as in the amino acid glycine) to a complex ring (as in the amino acid tryptophan).

A functional portion of an individual component
20 of the complexes may be defined here as a protein portion of an individual component of a complex still capable of forming a stable complex with another member of the complex under standard cellular and physiological conditions. For example, a functional portion of a component
25 may include, but is not limited to, a protein portion of dynamin which is still capable of stably binding a corresponding PYK2 polypeptide of an associated protein, and thus is still capable of forming a complex with that protein. Further, in the case of the catalytic domains
30 of the individual components of the invention, a functional portion of a catalytic domain may refer to a protein still capable of stably binding a substrate molecule under standard physiological conditions.

One method utilizing this approach that may be
35 pursued in the isolation of such complex component-binding molecules would include the attachment of a component molecule, or a functional portion thereof, to a solid

matrix, such as agarose or plastic beads, microtiter wells, petri dishes, or membranes composed of, for example, nylon or nitrocellulose, and the subsequent incubation of the attached component molecule in the presence of
5 a potential component-binding compound or compounds. Attachment to said solid support may be direct or by means of a component specific antibody bound directly to the solid support. After incubation, unbound compounds are washed away, component-bound compounds are recovered. By
10 utilizing this procedure, large numbers of types of molecules may be simultaneously screened for complex component-binding activity.

The complex components which may be utilized in the above screening method may include, but are not limited to, molecules or functional portions thereof, such as
15 catalytic domains, phosphorylation domains, extracellular domains, or portions of extracellular domains, such as ligand-binding domains, and adaptor proteins, or functional portions thereof. The peptides used may be phosphorylated, e.g., may contain at least one phosphorylated amino acid residue, preferably a phosphorylated Tyr amino acid residue, or may be unphosphorylated. A phosphorylation domain may be defined as a peptide region that is specifically phosphorylated at certain amino acid residues. A
20 functional portion of such a phosphorylation domain may be defined as a peptide capable of being specifically phosphorylated at certain amino acids by a specific protein.

Molecules exhibiting binding activity may be further screened for an ability to disrupt protein complexes. Alternatively, molecules may be directly screened
30 for an ability to promote the complexes. For example, in vitro complex formation may be assayed by, first, immobilizing one component, or a functional portion thereof, of the complex of interest to a solid support.
35 Second, the immobilized complex component may be exposed to a compound such as one identified as above, and to the second component, or a functional portion thereof, of the

complex of interest. Third, it may be determined whether or not the second component is still capable of forming a complex with the immobilized component in the presence of the compound. In addition, one could look for an increase
5 in binding.

Additionally, complex formation in a whole cell may be assayed by utilizing co-immunoprecipitation techniques well known to those of skill in the art. Briefly, a cell line capable of forming a complex of interest may
10 be exposed to a compound such as one identified as above, and a cell lysate may be prepared from this exposed cell line. An antibody raised against one of the components of the complex of interest may be added to the cell lysate, and subjected to standard immunoprecipitation techniques.
15 In cases where a complex is still formed, the immunoprecipitation will precipitate the complex, whereas in cases where the complex has been disrupted, only the complex component to which the antibody is raised will be precipitated.

20 A preferred method for assessing modulation of complex formation within a cell utilizes a method similar to that described above. Briefly, a cell line capable of forming a complex of interest is exposed to a test compound. The cells are lysed and the lysate contacted with
25 an antibody specific to one component of the complex, said antibody having been previously bound to a solid support. Unbound material is washed away, and the bound material is exposed to a second antibody, said second antibody binding specifically to a second component of the complex. The
30 amount of second antibody bound is easily detected by techniques well known in the art. Cells exposed to an inhibitory test compound will have formed a lesser amount of complex compared to cells not exposed to the test compound, as measured by the amount of second antibody
35 bound. Cells exposed to a test compound that promotes complex formation will have an increased amount of second antibody bound.

The effect of an agent on the differentiation capability of the complex of interest may be directly assayed. Such agents may, but are not required to, include those agents identified by utilizing the above screening technique. For example, an agent or agents may be administered to a cell such as a neuronal cell, capable of forming a complex, for example, which, in the absence of any agent, would not lead to the cell's differentiation. The differentiation state of the cell may then be measured either in vitro or in vivo. One method of measurement may involve observing the amount of neurile growth present.

Agents capable of disrupting complex formation and capable of reducing or inhibiting disorders, which involve the formation of such complexes, or which involve the lack of formation of such complexes, may be used in the treatment of patients exhibiting or at risk for such disorders. A sufficient amount of agent or agents such as those described above may be administered to a patient so that the symptoms of the disease or condition are reduced or eliminated.

XVII. Purification and Production of Complexes

Described in this Section are methods for the synthesis or recombinant expression of components, or fragments thereof, of the protein complexes of the invention. Also described herein are methods by which cells exhibiting the protein complexes of the invention may be engineered.

The complexes of the invention may be substantially purified, i.e., may be purified away from at least 90% (on a weight basis), and from at least 99%, if desired, of other proteins, glycoproteins, and other macromolecules with which it is associated. Such purification can be achieved by utilizing a variety of procedures well known to those of skill in the art, such as subjecting cells, tissue or fluid containing the complex to a combi-

nation of standard methods, for example, ammonium sulfate precipitation, molecular sieve chromatography, and/or ion exchange chromatography.

Alternatively, or additionally, a complex may be
5 purified by immunoaffinity chromatography using an immuno-
absorbent column to which an antibody is immobilized which
is capable of binding to one or more components of the
complex. Such an antibody may be monoclonal or poly-
clonal in origin. Other useful types of affinity purifi-
10 cation for the protein complex may utilize, for example,
a solid-phase substrate which binds the catalytic kinase
domain of a protein, or an immobilized binding site for
noncatalytic domains of the components of the complex,
which bind in such a manner as to not disrupt the complex.
15 The complex of the present invention may be biochemically
purified from a variety of cell or tissue sources.

Methods for the synthesis of polypeptides or
fragments thereof, which are capable of acting as compo-
nents of the complexes of the present invention, are well-
20 known to those of ordinary skill in the art. See, for
example, Creighton, Proteins: Structures and Molecular
Principles, W.H. Freeman and Co., NY (1983), which is
incorporated herein, by reference, in its entirety.

Components of a complex which have been sepa-
25 rately synthesized or recombinantly produced, may be
reconstituted to form a complex by standard biochemical
techniques well known to those skilled in the art. For
example, samples containing the components of the complex
may be combined in a solution buffered with greater than
30 about 150mM NaCl, at a physiological pH in the range of
7, at room temperature. For example, a buffer comprising
20mM Tris-HCl, pH 7.4, 137mM NaCl, 10% glycerol, 1% Triton
X-100, 0.1% SDS, 0.5% deoxycholate and 2mM EDTA could be
used.

35 Methods for preparing the components of complexes
of the invention by expressing nucleic acid encoding
proteins are described herein. Methods which are well

known to those skilled in the art can be used to construct expression vectors containing protein coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques and in vivo recombination/genetic recombination. DNA and RNA synthesis may, additionally, be performed using an automated synthesizers. See, for example, the techniques described in Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. (1989), and in Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y. (1989).

A variety of host-expression vector systems may be utilized to express the coding sequences of the components of the complexes of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, exhibit the protein complexes of the invention. These include but are not limited to microorganisms such as bacteria (e.g., E.coli, B. subtilis) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing protein coding sequences; yeast (e.g., Saccharomyces and Pichia) transformed with recombinant yeast expression vectors containing the protein coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the protein coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the protein coding sequences coding sequence; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian

cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems a number of expression
5 vectors may be advantageously selected depending upon the use intended for the complex being expressed. For example, when large quantities of complex proteins are to be produced for the generation of antibodies or to screen peptide libraries, vectors which direct the expression of
10 high levels of fusion protein products that are readily purified may be desirable. Such vectors include but are not limited to the E. coli expression vector pUR278 (Ruther et al., *EMBO J.* 2:1791, 1983), in which the protein coding sequence may be ligated individually into
15 the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye and Inouye, *Nucleic acids Res.* 13:3101-3109, 1985; Van Heeke & Schuster, *J. Biol. Chem.* 264:5503-5509, 1989); and the like. pGEX vectors may also be used to express foreign
20 polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX
25 vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned protein can be released from the GST moiety.

In an insect system, Autographa californica nuclear polyhidrosis virus (AcNPV) is used as a vector to
30 express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The complex coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin
35 promoter). Successful insertion of the PTK/adaptor complex coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant

virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted gene is expressed (e.g., see Smith et al., *J. Biol.* 46:584, 1983; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the complex coding sequence may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion into a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing proteins in infected hosts. (E.g., See Logan & Shenk, *Proc. Natl. Acad. Sci. USA* 81:3655-3659, 1984) Specific initiation signals may also be required for efficient translation of inserted coding sequences. These signals include the ATG initiation codon and adjacent sequences.

In cases where an entire protein gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription

enhancer elements, transcription terminators, etc. (see Bittner et al., *Methods in Enzymol.* 153:516-544, 1987)

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences; or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cells lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, etc.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably coexpress both the proteins may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the protein encoding DNA independently or coordinately controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker.

Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which coexpress both the PTK and

adaptor protein. Such engineered cell lines are particularly useful in screening and evaluation of compounds that affect signals mediated by the complexes.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al., *Cell* 11:223, 1977), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, *Proc. Natl. Acad. Sci. USA* 48:2026, 1962), and adenine phosphoribosyltransferase (Lowy et al., *Cell* 22:817, 1980) genes can be employed in tk⁻, hgp⁺ or aprt⁻ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler et al., *Natl. Acad. Sci. USA* 77:3567, 1980; O'Hare et al., *Proc. Natl. Acad. Sci. USA* 78:1527, 1981); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, *Proc. Natl. Acad. Sci. USA* 78:2072, 1981); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al., *J. Mol. Biol.* 150:1, 1981); and hyg⁺, which confers resistance to hygromycin (Santerre et al. *Gene* 30:147, 1984) genes.

New members of the protein families capable of forming the complexes of the invention may be identified and isolated by molecular biological techniques well known in the art. For example, a previously unknown protein encoding gene may be isolated by performing a polymerase chain reaction (PCR) using two degenerate oligonucleotide primer pools designed on the basis of highly conserved sequences within domains common to members of the protein family.

The template for the reaction may be cDNA obtained by reverse transcription of mRNA prepared from cell lines or tissue known to express complexes. The PCR product may be subcloned and sequenced to insure that the amplified sequences represent the sequences of a member of the PTK or adaptor subfamily. The PCR fragment may then be used to isolate a full length protein cDNA clone by radioactively labeling the amplified fragment and screen-

ing a bacteriophage cDNA library. Alternatively, the labeled fragment may be used to screen a genomic library. For a review of cloning strategies which may be used. See e.g., Maniatis, Molecular Cloning: A Laboratory Manual,
5 Cold Springs Harbor Press, N.Y. (1989); and Ausubel et al., Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989). A general method for cloning previously unknown proteins has been described by Skolnik (Skolnik, E.Y., Cell 65:75,
10 1991) and Skolnik et al., (U.S. Patent Application Serial No. 07/643,237) which are incorporated herein, by reference, in their entirety, including drawings.

XVIII. Derivatives of Complexes

Also provided herein are functional derivatives
15 of a complex. By "functional derivative" is meant a "chemical derivative," "fragment," "variant," "chimera," or "hybrid" of the complex, which terms are defined below. A functional derivative retains at least a portion of the function of the protein, for example reactivity with an
20 antibody specific for the complex, enzymatic activity or binding activity mediated through noncatalytic domains, which permits its utility in accordance with the present invention.

A "chemical derivative" of the complex contains
25 additional chemical moieties not normally a part of the protein. Such moieties may improve the molecule's solubility, absorption, biological half life, and the like. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any
30 undesirable side effect of the molecule, and the like. Moieties capable of mediating such effects are disclosed in Remington's Pharmaceutical Sciences (1980). Procedures for coupling such moieties to a molecule are well known in the art. Covalent modifications of the protein complex or
35 peptides are included within the scope of this invention. Such modifications may be introduced into the molecule by

reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues, as described below.

5 Cysteiny l residues most commonly are reacted with alpha-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteiny l residues also are derivatized by reaction with bromotri-
10 fluoroacetone, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide; p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction
15 with diethylprocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Parabromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysiny l and amino terminal residues are reacted
20 with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysiny l residues. Other suitable reagents for derivatizing primary amine containing residues include imidoesters such as methyl
25 picolinimide; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginy l residues are modified by reaction with
30 one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group.
35 Furthermore, these reagents may react with the groups of lysine as well as the arginine alpha-amino group.

Tyrosyl residues are well-known targets of modification for introduction of spectral labels by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizol and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction carbodiimide ($R'-N-C-N-R'$) such as 1-cyclohexyl-3-(2-morpholinyl(4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residue are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Derivatization with bifunctional agents is useful, for example, for cross-linking the component peptides of the complexes to each other or the complex to a water-insoluble support matrix or to other macromolecular carriers. Commonly used cross-linking agents include, for example, 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[p-azidophenyl] dithiolpropioimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains
5 (Creighton, T.E., *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the Nterminal amine, and, in some instances, amidation of the C-terminal carboxyl groups.

Such derivatized moieties may improve the
10 stability, solubility, absorption, biological half life, and the like. The moieties may alternatively eliminate or attenuate any undesirable side effect of the protein complex and the like. Moieties capable of mediating such effects are disclosed, for example, in Remington's Pharma-
15 ceutical Sciences, 18th ed., Mack Publishing Co., Easton, PA (1990).

The term "fragment" is used to indicate a polypeptide derived from the amino acid sequence of the proteins, of the complexes having a length less than the
20 full-length polypeptide from which it has been derived. Such a fragment may, for example, be produced by proteolytic cleavage of the full-length protein. Preferably, the fragment is obtained recombinantly by appropriately modifying the DNA sequence encoding the proteins to delete
25 one or more amino acids at one or more sites of the C-terminus, N-terminus, and/or within the native sequence. Fragments of a protein, when present in a complex resembling the naturally occurring complex, are useful for screening for compounds that act to modulate signal
30 transduction, as described below. It is understood that such fragments, when present in a complex may retain one or more characterizing portions of the native complex. Examples of such retained characteristics include: catalytic activity; substrate specificity; interaction
35 with other molecules in the intact cell; regulatory functions; or binding with an antibody specific for the native complex, or an epitope thereof.

Another functional derivative intended to be within the scope of the present invention is a complex comprising at least one "variant" polypeptide which either lack one or more amino acids or contain additional or substituted amino acids relative to the native polypeptide. The variant may be derived from a naturally occurring complex component by appropriately modifying the protein DNA coding sequence to add, remove, and/or to modify codons for one or more amino acids at one or more sites of the C-terminus, N-terminus, and/or within the native sequence. It is understood that such variants having added, substituted and/or additional amino acids retain one or more characterizing portions of the native complex, as described above.

A functional derivative of complexes comprising proteins with deleted, inserted and/or substituted amino acid residues may be prepared using standard techniques well-known to those of ordinary skill in the art. For example, the modified components of the functional derivatives may be produced using site-directed mutagenesis techniques (as exemplified by Adelman et al., 1983, DNA 2:183) wherein nucleotides in the DNA coding the sequence are modified such that a modified coding sequence is modified, and thereafter expressing this recombinant DNA in a prokaryotic or eukaryotic host cell, using techniques such as those described above. Alternatively, components of functional derivatives of complexes with amino acid deletions, insertions and/or substitutions may be conveniently prepared by direct chemical synthesis, using methods well-known in the art. The functional derivatives of the complexes typically exhibit the same qualitative biological activity as the native complexes.

XIX. Evaluation of Disorders

The protein complexes of the invention involved in disorders may be utilized in developing a prognostic evaluation of the condition of a patient suspected of

exhibiting such a disorder. For example, biological samples obtained from patients suspected of exhibiting a disorder involving a protein complex may be assayed for the presence of such complexes. If such a protein complex is normally present, and the development of the disorder is caused by an abnormal quantity of the complex, the assay should compare complex levels in the biological sample to the range expected in normal tissue of the same cell type.

Among the assays which may be undertaken may include, but are not limited to isolation of the protein complex of interest from the biological sample, or assaying for the presence of the complex by exposing the sample to an antibody specific for the complex, but non-reactive to any single, non-complexed component, and detecting whether antibody has specifically bound.

Alternatively, one or more of the components of the protein complex may be present in an abnormal level or in a modified form, relative to the level or form expected in a modified form, relative to the level or form expected in a normal, nononcogenic tissue of the same cell type. It is possible that overexpression of both components may indicate a particularly aggressive disorder. Thus, an assessment of the individual and levels of mRNA and protein in diseased tissue cells may provide valuable clues as to the course of action to be undertaken in treatment of such a disorder. Assays of this type are well known to those of skill in the art, and may include, but are not limited to, Northern blot analysis, RNase protection assays, and PCR for determining mRNA levels. Assays determining protein levels are also well known to those of skill in the art, and may include, but are not limited to, Western blot analysis, immunoprecipitation, and ELISA analysis. Each of these techniques may also reveal potential differences in the form (e.g., the primary, secondary, or tertiary amino acid sequence, and/or post-translational modifications of the sequence) of the component(s).

Examples

The examples below are non-limiting and are merely representative of various aspects and features of the procedures used to identify the full-length nucleic acid and amino acid sequences of PYK-2. Experiments demonstrating PYK-2 expression, interaction and signalling activities are also provided.

Materials and Methods

Chemicals

Bradykinin, pertusis toxin, cholera toxin, forskolin, phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187, carbachol, muscarine, atrophine, mecamlamine, and 1,1-dimethyl-4-phenyl piperazinium iodide (DMPP) were purchased from Sigma.

Cloning of PYK2 cDNA

We have used the Grb2 adaptor protein as a specific probe for screening-expression libraries in order to isolate Grb2 binding proteins. One of the cloned proteins encoded a protein tyrosine kinase that contains a proline rich region that can bind in vitro to the SH3 domains of Grb2. This protein was termed PYK1 for proline rich tyrosine kinase 1. Comparison of the amino acid sequence of PYK1 to other tyrosine kinases, indicated that PYK1 is related to the Ack protein tyrosine kinase. Analysis of PYK1 sequence indicated that this kinase represents a new class of cytoplasmic protein tyrosine kinases.

In an attempt to isolate kinases related to PYK1, we applied the polymerase chain reaction (PCR) utilizing degenerate oligonucleotide primers, derived from PYK1 sequence according to the conserved motifs of the catalytic domains of PTKs. RNA from rat spinal cord was used to prepare cDNA utilizing the reverse transcriptase of Molony murine leukemia virus (BRL) according to the manufacturer's protocol. The cDNA was amplified by PCR

utilizing degenerate oligonucleotides primers corresponding to conserved tyrosine kinase motifs from subdomains TK6 and TK9 of PYK1; (the sense and antisense primers correspond to amino acid sequences IHRDLAARN [SEQ. ID NO 3] and WMFGVTLW [SEQ. ID NO 4] respectively). The PCR was carried out under the following conditions; 1 min at 94°C; 1 min at 50°C and 1 min at 68°C for 35 cycles. PCR products were electrophoresed, checked by the size (~210bp), purified and subcloned into pBluescript (Stratagene). Novel clones were screened by DNA sequencing. The cDNA insert of clone #38 was used as probe to screen human brain cDNA libraries (human fetal brain λ gt 10 and human brain λ gt 11, 6×10^5 recombinant clones each) essentially as described by Maniatis ().

The complete amino acid sequence of a novel protein tyrosine kinase was isolated from human brain cDNA library and termed PYK2. The open reading frame of PYK2 encodes a protein of 1009 amino acids containing a long N-terminal sequence of 424 amino acids followed by a protein tyrosine kinase domain, two proline rich domains (29% and 23.3% proline respectively) and a large carboxy terminal region. The kinase domain of PYK2, contains several sequence motifs conserved among protein tyrosine kinases, including the tripeptide motif DFG, found in most kinases, and a consensus ATP binding motif GXGXXG followed by AXK sequence 17 amino acids residues downstream.

Comparison of the amino acid sequence of the kinase domain of PYK2 with other protein tyrosine kinases showed that the kinase core of PYK2 is most similar to the protein tyrosine kinase domains of Fak, Fer, Her4 and Abl. In addition to the sequence homology in the kinase domain, the flanking sequences and the overall structural organization of the PYK2 protein are similar to those of FAK indicating that PYK2 belong to the same family of non-receptor similar to those of Fak protein tyrosine kinases.

DNA Sequencing and Analysis

DNA sequencing was performed on both strands utilizing series of oligonucleotide primers and subclones. The nucleotide sequence and the deduced amino acid
5 sequence were subjected to homology search with Genbank and PIR databases using FASTA and BLAST mail-server program.

Northern blot analysis

Total RNA was isolated from mouse tissues by the
10 acid guanidinium thiocyanate-phenol-chloroform method (Anal. Biochem. 162; 156, 1987). Poly (A)⁺ RNA was denatured with formaldehyde and electrophoresed on a 1% agarose/0.7% formaldehyde gel. RNAs were transferred to a nitrocellulose membrane and hybridized with ³²P-labeled
15 probe that contained the cDNA insert of clone #38 as described above.

Antibodies

Antibodies against PYK2 were raised in rabbits immunized (HTI) either by GST fusion protein containing
20 residues 362-647 or PYK2 or by synthetic peptide corresponding the 15 amino acids at the N-terminal end of PYK2. Antisera were checked by immunoprecipitation and immunoblot analysis, and the specificity was confirmed either by reactivity to the related protein Fak or by
25 competition with the antigenic or control peptides.

Antibodies against PYK-2 were raised in rabbits immunized either with GST fusion protein containing residues of PYK-2 or with synthetic peptide corresponding the 15 amino acids at the N-terminal end of PYK-2. The
30 antibodies are specific to PYK-2 and they do not cross react with FAK.

Cells and cell culture

PC12-rat pheochromocytoma cells were cultured in Dulbecco's modified Eagle's medium containing 10% horse

serum, 5% fetal bovine serum, 100 μ g/ml streptomycin and 100 units of penicillin/ml. NIH3T3, 293, GP+E-86 and PA317 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 μ g/ml streptomycin and 100 units of penicillin/ml.

Transfections and infections

For stable expression in PC12 cells, PYK2 was subcloned into the retroviral vector pLXSN (Miller and Rosman, Biotechniques 7:980, 1989). The construct was used to transfect GP+E-86 cells using lipofectimine reagent (GIBCO BRL). 48 hours after transfection, virus containing supernatants were collected. Pure retrovirus-containing cell-free supernatant were added to PC12 cells in the presence of polybrene (8 μ g/ml, Aldrich) for 4 hours (MCB 12 491, 1992). After 24 hours, infected PC12 cells were split into growing medium containing 350 μ l/mg G418. G418 resistant colonies were isolated two to three weeks later and the level of expression was determined by western blot analysis.

Stable cell lines of NIH3T3 that overexpress PYK2 were established by cotransfection of PYK2 subcloned into pLSV together with pSV2neo utilizing lipofectamine reagent (GIBCO BRL). Following transfection the cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1mg/ml G418. Transient transfections into 293 cells were performed by using the calcium phosphate technique (*).

Constructs

GST-PYK2- a DNA fragment of λ 900bp corresponding to residues 362-647 of PYK2 was amplified by PCR utilizing the following oligonucleotide primers: 5'-CGGGATCCTCATCATCCATCCTAGGAAAGA-3' (sense) [SEQ. ID NO 5] and 5'-CGGGAATTCGTCGTAGTCCCAGCAGCGGGT-3' (antisense) [SEQ. ID NO 6].

The PCR produce was digested with HamHI and ECORI and subcloned into pGEX3X (Pharmacia). Expression of GST-PYK2 fusion protein was induced by the 1mM IPTG essentially as described by Smith et al., (Gene 67:31, 5 1988). The fusion protein was isolated by electroelution from SDS-PAGE.

PYK2- The full length cDNA sequence of PYK2 was subcloned into the following mammalian expression vectors: pLSV; downstream the SV40 early promoter, pLXSN-retroviral 10 vector; downstream the Mo-MuLV long terminal repeat; pRK5; downstream the CMV promoter.

PYK2-HA- the influenza virus hemagglutinin peptide (YPYDVDPYAS) [SEQ. ID NO 7] was fused to the C-terminal end of PYK2 utilizing the following 15 oligonucleotide primers in the PCR: 5'-CACAATGTCTTCAAACGCCAC-3' [SEQ. ID NO 8] and 5'-GGCTCTAGATCACGATGCGTAGTCAGGGACATCGTATGGGRACTCTGCAGGTGGGTGGGCCAG-3'. [SEQ. ID NO 9].

The amplified fragment was digested with RsrII and XbaI and used to substitute the corresponding fragment of PYK2. 20 The nucleotide sequence of the final construct was confirmed by DNA sequencing.

Kinase negative mutant- in order to construct a kinase negative mutant, Lys (457) was substituted to Ala 25 by site directed mutagenesis utilizing the 'Transformer Site-Directed Mutagenesis Kit' (Clontech). The oligonucleotide sequence was designed to create a new restriction site of NruI. The nucleotide sequence of the mutant was confirmed by DNA sequencing. The 30 oligonucleotide sequence that used for mutagenesis is: 5'-CAATGTAGCTGTCGCGACCTGCAAGAAAGAC-3' [SEQ. ID NO 10] (NruI site - bold, Lys-AAC substituted to Ala-GCG underline).

Rak-HA- The rak cDNA subcloned in pBluescript was 35 obtained from Bernardu Rudi (NYU medical center). The influenza virus hemagglutinin peptide was fused to the C-terminal end of Rak essentially as described for PYK2.

The oligonucleotide primers that were used in the PCR were: 5'-GCCAGCAGGCCATGTCACTGG-3' [SEQ. ID NO 11] and 5'-CGGAATTCTTACGATGCGTAGTCAGGGACATCGTATG GGTAGACATCAGTTAACATTTTG-3'. [SEQ. ID NO 12]

- 5 The pcr product was digested with ball and EcoRI and was used to substitute the corresponding fragment at the C-terminal end of Rak. The Rak-HA cDNA was subcloned into PRK5 downstream the DMV promotor and into the retroviral vector pLXSN, downstream the Mo-MuLV long terminal repeat.

10 Immunoprecipitation and Immunoblot Analysis

- Cell were lysed in lysis buffer containing 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulferic acid (HEPES pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM ethyleneglycol-bis (β -aminoethyl ether)-
- 15 N,N,N'-teraacetic acid (EGTA), 10 μ g leupeptin per ml, 10 μ g aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride (PMSF), 200 μ M sodium orthovanadate and 100 mM sodium fluoride. Immunoprecipitations were performed using protein A-sepharose (Pharmacia) coupled to specific
- 20 antibodies. Immunoprecipitates were washed either with HNTG' solution (20 mM HEPES buffer at pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 100 mM sodium fluoride, 200 μ M sodium orthovanadate) or successively with H' solution (50 mM Tris-HCl pH8, 500 mM NaCl, 0.1% SDS, 0.2%
- 25 Triton X-100, 100 mM NaF, 200 μ M sodium orthovanadate) and L' solution (10 mM Tris-HCl pH 8, 0.1% Triton X-100, 100 mM NaF, 200 μ M sodium orthovanadate).

- The washed immunoprecipitates incubated for 5 min with gel sample buffer at 100°C and analyzed by sodium
- 30 dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In some experiments the gel-embedded proteins were electrophoretically transferred onto nitrocellulose. The blot was then blocked with TBS (10 mM Tris pH 7.4, 150 mM NaCl) that contained 5% low fat milk and 1% ovalbumin.
- 35 Antisera or purified mAbs were then added in the same solution and incubation was carried out for 1 h at 22°C.

For detection the filters were washed three times (5 min each wash) with TBS/0.05% Tween-20 and reacted for 45 min at room temperature with horseradish peroxidase-conjugated protein A. The enzyme was removed by washing as described
5 above, and the filters were reacted for 1 min with a chemiluminescence reagent (ECL, Amersham) and exposed to an autoradiography film for 1-15 min.

In vitro kinase assay

This was carried out on immunoprecipitates in 50
10 μ l HNTG (20 mM Hepes pH 7.5, 150 mM NaCl, 20% glycerol, 0.1% Triton X-100) containing 10 mM MnCl₂ and 5 μ Ci or [mN-³²P]ATP for 20 min at 22°C. The samples were washed with H', M' and, L' washing solutions, boiled for 5 min in sample buffer and separated by SDS-PAGE.

15 Isolation of ACK/PYK

ACK/PYK may be isolated as described in Manser et al., Nature, 363:364-367, 1993. Comparison analysis of the full length sequence of ACK/PYK with other tyrosine kinases indicates that is not closely related to any of
20 these, although it has some similarity to the focal adhesion kinase. Therefore, ACK/PYK represents a separate class of tyrosine kinases and isolation of related genes that belong to the same class is a major accomplishment.

Example 1: Isolation of PYK-2 cDNA

25 To identify genes related to the ACK/PYK protein tyrosine kinase, the polymerase chain reaction (PCR) was applied in combination with degenerated oligonucleotide primers based upon conserved motifs of the kinase domain of PTKs.

30 Oligonucleotides primers specifically designed to a highly conserved N-terminal motif of PTKs within subdomain TK6 (IHRDLAARN) SEQ ID NO 13. and ACK/PYK specific C-terminal primers within subdomain TK9 (WMFGVTLW) SEQ ID NO 14 were utilized. The amplification

reactions with cDNA templates from 8 different sources gave rise to fragments of 0.2-0.9kb. The PCR products were subcloned into pBlueScript and screened by DNA sequencing and hybridization under low stringency conditions.

A cDNA fragment of 210bp from rat spinal cord was identified which is highly related to the Focal Adhesion Kinase (FAK). The fragment was sequenced in the 3' and 5' directions and was subsequently used as a probe to screen cDNA libraries (human fetal brain λ gt 10 and human brain λ gt 11, 6×10^5 recombinant clones each).

Several overlapping clones spreading 1.5-3kb were isolated and their cDNA inserts were analyzed by PCR, restriction mapping and sequencing. Two clones (#1 and #11) were chosen for further analysis and subcloning. Clone #1 contains an insert of 2.7kb from the 5' end of the gene, and clone #11 contains an insert of 3kb from the 3' end of the gene.

By utilizing a series of subclones and synthesized oligonucleotide primers the full length sequence of PYK-2 was determined. The sequence analysis resulted in a composite sequence of 3309bp long which contains a 104 bp 5' untranslated region, a 3021 bp coding region and 184 bp 3' untranslated region. The ATG encoding the translation initiation codon is preceded by four translation stop codons in all reading frames.

The long open reading frame encodes a protein of 1007 amino acids (predicted molecular mass of 110,770d) whose structural organization is very similar to FAK. The PYK-2 protein contains a long N-terminal sequence of 422 amino acids followed by a tyrosine kinase catalytic domain. The PYK-2 protein also contains the structural motifs common to all PTKs, two proline rich domains (19.6% and 17% proline respectively) and a focal adhesion targeting (FAT) motif in the C-terminal end. Comparison analysis of the amino acid sequence of PYK-2 with the human FAK revealed 52% identity between the two proteins.

The kinase domain and the FAT sequence are most closely related (62% homology).

The PYK-2 protein contains several predicted binding sites for intracellular substrates. For example, 5 YLMV [SEQ. ID NO 15] is a predicted binding site for GRB2 SH2 domain - tyrosine 879 of PYK-2. YVVV [SEQ. ID NO 16] is a predicted binding site for SHPTP2 - tyrosine 903 of PYK-2. There are predicted phosphorylation sites for PKC, PKA and Ca/Calmodulin kinase. In addition, tyrosine 402 10 is a predicted autophosphorylation site of PYK-2 and it may be involved in the binding of a src SH2 domain. This is based on the homology between tyrosine 397 of FAK which was mapped as a major autophosphorylation site both in vivo and in vitro. This tyrosine provides an high 15 affinity binding site for a src SH2 domain. Both tyrosine 397 of FAK and tyrosine 402 of PYK-2 are located at the juncture of the N-terminal and the catalytic domain and are followed by sequence (Y)AEI which is very similar to the consensus of the high affinity src SH2 domain binding 20 peptide YEEI.

Total RNA from rat spinal cord was used to prepare cDNA utilizing the reverse transcriptase of Molony murine leukemia virus ('Superscript', BRL) according to the manufacturer's protocol. The cDNA was amplified by 25 PCR utilizing degenerate oligonucleotides primers corresponding to conserved tyrosine kinase motifs from subdomains TK6 and TK9 of PYK1; (the sense and antisense primers correspond to amino acid sequences IHRDLAARN [SEQ. ID NO 17] and WMFGVTLW [SEQ. ID NO 18] respectively). The 30 PCR was carried out under the following conditions; 1 min at 94°C; 1 min at 50°C and 1 min at 68°C for 35 cycles. Amplified DNA was subcloned and sequence, resulting in identification of a novel tyrosine kinase termed PYK2. A λ gt10 human fetal brain cDNA library (clontech) was 35 screened with ³²P-labeled PCR clone corresponding to rat PYK2. Four overlapping clones were isolated, their DNA sequence was determined on both strands utilizing series

of oligonucleotide primers. The 314-bp consensus sequence contains a single open reading frame of 3027 nucleotide preceded by a 105 nucleotide 5'-untranslated region. Amino acid sequence comparisons were performed using, the Smith-Waterman algorithm of MPSRCH (IntelliGenetic) on MasPar computer.

The deduced amino acid sequence of human PYK2 from CDNA clones is shown in Figure 4. The tyrosine kinase domain is highlighted by a dark shaded box. Two proline-rich domains in the C-terminal region are boxed with light shading. Amino acid residues are numbered on the left. Comparison of the amino acid sequence of the catalytic domain of PYK2 with the four most related human protein tyrosine kinases demonstrated 61%, 43%, 40% and 41% sequence identity between PYK2 and Fak, Fer, HER4 and Abl, respectively. The homology between PYK2 and Fak extends beyond the catalytic domain with 42% and 36% amino acid identity in the N-terminal and C-terminal regions, respectively.

20 Example 2: Pattern of PYK-2 Expression

PYK2 is highly expressed in the nervous system. We examined the expression pattern and the tissue distribution of PYK2 by Northern blot and by *in situ* hybridization analyses.

25 The tissue distribution of PYK-2 expression was determined by Northern blot analysis. Poly(A)⁺ RNAs were purified from mouse tissues (liver, lung, spleen, kidney, heart, brain, skin, uterus) and hybridized with two different probes corresponding to two different regions of the PYK-2 gene. The results were identical in both cases. 30 A 4.2-4.5kb PYK-2 transcript is relatively abundant in the brain but was also found in lower levels in the spleen and in the kidney.

Film autoradiography of a sagittal section 35 through the adult rat brain shows very high levels of expression in the olfactory bulb (OB), hippocampus (Hi),

and dentate gyrus (DG). Moderate levels of expression are seen in the cerebral cortex (Cx), striatum (S), and thalamus (T). Low levels of expression are seen in the cerebellum (Cb) and brainstem (BS).

5 Expression of PYK2 mRNA determined by Northern blot analysis of poly(A)+ from various human tissues. The northern blot was hybridized with 3.9kb ³²P-labeled fragment containing the PYK2 cDNA in 50% formamide at 42°C.

 Northern blot of mRNA isolated from various human
10 brain sections (amygdala, caudate nucleus, corpus callosum, hippocampus, hypothalamus, substantia nigra, subthalamic nucleus and thalamus) revealed highest expression in the hippocampus and amygdala, moderate level of expression in the hypothalamus, thalamus and caudate
15 nucleus and low level of expression in the corpus callosum and subthalamic nucleus.

 These results are consistent with *in situ* hybridization analysis on day 7 post natal rat brain sections utilizing antisense probes derived from PYK2
20 sequence. The *in situ* hybridization analysis demonstrate that the olfactory bulb, the hippocampus and the dentate gyrus exhibit high level of PYK2 transcripts. Moderate levels of PYK2 expression was detected in the striatum, cerebral cortex and thalamus and low levels of expression
25 was detected in the cerebellum and brainstem.

 In order to characterize the PYK2 protein, NIH3T3 cells were transfected with a mammalian expression vector that encodes PYK2 protein with an influenza virus hemagglutinin peptide tag. PYK2 was immunoprecipitated
30 with either anti-PYK2 or anti-HA antibodies from 3T3 transfected cell, whereas the endogenous PYK2 protein was immunoprecipitated with anti-PYK2 antibodies from PC12 cells. These antibodies precipitated a protein that migrated in SDS gels with apparent molecular weight of 112
35 kDa. Addition of Y-[³²p]ATP to immunoprecipitates from PYK2 transfected cells followed by SDS-PAGE analysis and

autoradiography showed that PYK2 undergoes phosphorylation on tyrosine residues.

The expression of PYK-2 in different cell lines was analyzed by IP/IB utilizing anti-PYK-2 antibodies directed to the kinase domain as described previously (GST-PYK-2). The expression pattern is summarized in table 1. Some of the interesting observations are a mobility shift of PYK-2 after differentiation of CHRF and L8057 (premegakaryocyte cell lines) by TPA, high expression of Fak and PYK-2 in different cell lines; and in XC cells (rat sarcoma) PYK-2 is phosphorylated on tyrosine.

PYK2 was immunoprecipitated from NIH3T3 cells, NIH3T3 cells that overexpress PYK2-HA and PC12 cells. The immunocomplexes were washed and resolved by 7.5% SDS-PAGE. immunoblotting was performed with anti-PYK2 antibodies. In vitro Kinase activity of PYK2. Cos cells were transiently transfected with PYK2-HA expression vector (+) or with an empty vector (-). The PYK2 protein was immunoprecipitated with anti-HA antibodies, the immunocomplexes were washed and subjected to in vitro kinase assay.

In situ hybridization was performed as follows: Fresh frozen rat brains were cut on a cryostat into 20-mm thick sections and thaw-mounted onto gelatin coated slides. The sections were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate (pH=7.4) for 30 minutes and rinsed three times for 5 minutes each in PBS and one time for 10 minutes in 2 X SSC. Two probes were used in the hybridization analysis, a 51 base oligonucleotide complementary to the sequence encoding amino acid 301-317, and a 51 base oligonucleotide complementary to the sequence encoding, amino acid 559-575 (from rat PCR product).

The oligonucleotides were labeled with α -³⁵S dATP (Du Pont-New England Nuclear) using terminal deoxynucleotidyl-transferase (Boehringer Mannheim) and

purified using sephadex G-25 quick spin columns (Boehinger Mannheim). The specific activity of the labeled probes was between 5×10^8 and 1×10^9 cpm/mg. Prehybridization and hybridization were carried out in a buffer containing
5 50% deionized formamide, 4 X SSC, 1 X Denhardts' solution, 500 ug/ml denatured salmon sperm DNA, 250 ug/ml yeast tRNA, and 10% dextran sulfate. The tissue was incubated for 12 hours at 45°C in hybridization solution containing the labeled probe (1×10^6 cpm/section) and 10 mM
10 dithiothreitol.

Controls for specificity were performed on adjacent sections by competitively inhibiting hybridization of the labeled oligonucleotides with a 30-fold concentration of unlabeled oligonucleotide and by
15 hybridization with sense probes. After hybridization, the sections were washed in two changes of 2 X SSC at room temperature for 1 hour, 1 X SSC at 55°C for 30 minutes, 0.5 X SSC at 55°C for 30 minutes, and 0.5 X SSC at room temperature for 15 minutes and then dehydrated in 60, 80,
20 95, and 100% ethanol. After air drying, the sections were exposed to x-ray film for 5 days. The sections were then dipped in Ilford K.5 photographic emulsion (Polysciences), exposed for 4 weeks at 4°C, and developed using Kodak D-19 developer and rapid fixer.

25 Emulsion autoradiography was examined by dark-field microscopy on a Zeiss axioskop. The influenza virus hemagglutinin peptide (YPYDVDPYAS) [SEQ. ID NO 19] tag was added to the C-terminal end of PYK2 utilizing the following oligonucleotide primers in the PCR: '5-
30 CACAATGTCTTCAAACGCCAC'3' [SEQ. ID NO 20] and '5GGCTCTAGATCACGATGCGTAGTCAGGGACATCGTATGGGTACTCTGCAGG TGGGTGGGCCAG-'3' [SEQ. ID NO 21]. The amplified fragment was digested with RsrII and XbaI and used to substitute the corresponding fragment of PYK2. The nucleotide
35 sequence of this construct was confirmed by DNA sequencing.

In vitro kinase assay was carried out on immunoprecipitates in 50 μ l HNTG (20 mM Hepes pH 7.5, 150 mM NaCl, 20% glycerol, 0.1% Triton X-100) containing 10 mM MnCl₂ and 5 mCi of [λ -³²P] ATP for 20 min at 22°C. The samples were washed with H' (50 mM Tris-HCl pH 8, 500 mM NaCl, 0.1% SDS, 0.2% Triton X-100, 5 mM EGTA, 100 mM NaF, 200 μ M sodium orthovanadate), M' (50 mM Tris-HCl pH 8, 150 mM NaCl, 7.5 mM EDTA, 0.1% SDS, 0.2% Triton X-100, 100 mM NaF, 200 μ M sodium orthovanadate) and L' (10 mM Tris-HCl pH 8, 0.1% Triton X-100, 100 mM NaF, 200 μ M sodium orthovanadate) washing, solutions, boiled for 5 min in sample buffer and separated by SDS-PAGE.

Antibodies against PYK2 were raised in rabbits immunized with GST fusion protein containing residues 62-647 of PYK-2. Antibodies against influenza virus hemagglutinin peptide were purchased from Boehringer Mannheim. Cell lysis, immunoprecipitations and immunoblotting was performed essentially as described by Lev et al. Mol. Cell. Biol. 13, 2224-2234, 1993.

Example 3: Properties of PYK-2 protein

In order to analyze the biochemical properties of PYK-2 the full length cDNA was subcloned into the two mammalian expression vectors RK5 and pLSV. In parallel, an expression vector encoding the PYK-2 protein fused to the influenza virus hemagglutinin peptide was constructed. This construct was used to identify the protein utilizing anti-HA antibodies.

pLSV-PYK-2-HA was transfected into cos cells. The protein was expressed at the predicted molecular mass (~116kD) as determined by IP and IB with anti-HA antibodies. The protein is an active kinase as determined by in vitro kinase assay utilizing (λ ³²P) ATP or an in vitro kinase assay utilizing cold ATP and immunoblotting with anti-phosphotyrosine antibodies.

The PYK-2 cDNA cloned in pLSV was cotransfected with pSV2neo into PC12 cells and NIH3T3 in order to

establish stable cell lines. G418 resistant colonies were screened by immunoprecipitating and immunoblotting.

NIH3T3 cell lines were established that overexpress the PYK-2 and the PYK-2-HA protein. In these
5 cells PYK-2 undergoes tyrosine phosphorylation in response to PDGF, EGF and aFGF. The level of phosphorylation is not so high. The stronger effect is achieved by TPA treatment (6 μ M) after 15 min incubation at 37°C as determined by time course analysis.

10 Example 4: Tyrosine phosphorylation of PYK2 in response to carbachol, membrane depolarization and Ca²⁺ influx.

The phosphorylation of PYK-2 on tyrosine residue in response to different stimulus was analyzed by immunoprecipitation of PYK-2 and immunoblotting with anti-
15 phosphotyrosine antibodies and vice versa.

The following treatments were used: Bradykinin, TPA, forskolin, forskolin + TPA, bradykinin + forskolin, NGF, Neuropeptide Y, Cholera toxin, Cholera toxin+TPA, Cholera toxin + bradykinin, pertusis toxin, pertusis toxin
20 + TPA, bradykinin + pertusis toxin, calcium ionophore A23187, bombesin.

The following results were obtained: PYK-2 undergoes tyrosine phosphorylation in response to TPA (1.6 μ M 15 min at 37°C), bradykinin (1 μ M 1 min at 37°C) and
25 calcium ionophore A23187 (2 μ M 15 min at 37 °C). Forskolin increase the response of TPA but does not give any signal by itself. Cholera toxin gave higher signal in combination with TPA and bradykinin but didn't cause phosphorylation of PYK-2 alone. Pertusis toxin also
30 induced the response of TPA and bradykinin but didn't cause any response alone. In order to determined if the bradykinin effect is mediated by PKC signaling pathway attempts to down regulate PKC by chronic treatment with TPA (twice) did not give a clear answer.

35 One interpretation of these results is that PKC and PKA (and maybe Ca/calmodulin kinase) induce the

autophosphorylation of PYK-2 in response to ser/the phosphorylation. This interpretation may be checked by utilizing specific inhibitors to PKC and PKA and by phosphamino-acid analysis.

5 Confluent PC12 cells in 150mm plates were grown for 18 hours in DMEM containing 0.5% horse serum and 0.25% fetal bovine serum. The cells were stimulated at 37°C with different agonists as indicated. washed with cold PBS and lysed in 800ml lysis buffer (Lev et al., supra).

10 The cell lysates were subjected to immunoprecipitation with anti-PYK2 antibodies. Following SDS-PAGE and transfer to nitrocellulose, the samples were immunoblotted with either antiphosphotyrosine (RC20, transduction laboratories) or anti-PYK2 antibodies.

15 Carbachol induces tyrosine phosphorylation of PYK2 via activation of the nicotinic acetylcholine receptor. Immunoprecipitates of PYK2 from PC12 cells that were subjected to the following treatments: muscarine (1mM) or carbachol (1mM) for 20 sec at 37°C.

20 Carbachol(1mM), DMPP (100μM), or carbachol after pretreatment with the muscarinic antagonist atropine (100nM) or the nicotinic antagonist mecamylamine (10μM) for 5 min at 37°C. Incubation with carbachol in the presence or absence of EGTA (3mM) as indicated. The

25 immunocomplexes were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with either anti-phosphotyrosine antibodies or with anti-PYK2 antibodies as indicated. Membrane depolarization and calcium ionophore induce tyrosine phosphorylation of PYK2.

30 Immunoprecipitates of PYK2 from quiescent PC12 cells were subjected to the following treatments: incubation with 75mM KCl in the presence or absence of EGTA (3mM), incubation with 6μM of the calcium ionophore A23187 for 15 min at 37°C. The immunoprecipitates were washed, resolved

35 by 7.5% SDS-PAGE and immunoblotted with either anti-phosphotyrosine antibodies or with anti-PYK2 antibodies.

Activation of PYK2 by carbachol, membrane depolarization and Ca^{+2} influx was studied. Since PYK2 is highly expressed in the central nervous system and in PC12 cells, we examined the effect of a variety of neuronal agonists on the phosphorylation state of PYK2. In these experiments, PC12 cells were treated with an agonist, lysed and subjected to immunoprecipitation with anti-PYK2 antibodies followed by SDS-PAGE analysis and immunoblotting with phosphotyrosine specific antibodies.

Stimulation of PC12 cells with carbachol induces strong, tyrosine phosphorylation of PYK2. We explored the possibility whether activation of both cholinergic receptor subtypes leads nicotinic and muscarinic receptors to tyrosine phosphorylation of PYK2. Pharmacological analysis with either subtype specific agonists, muscarine and DMPP or subtype specific antagonists, atropine and mecamylamine indicated that activation of PYK2 by carbachol is mediated via the nicotinic acetylcholine receptor. The phosphorylation of PYK2 in response to carbachol is very rapid; 5 second after applying carbachol to the cells, PYK2 became phosphorylated on tyrosine residues. Elimination of extracellular calcium by EGTA completely blocked agonist induced tyrosine phosphorylation of PYK2, indicating that calcium influx is required for carbachol induced PYK2 activation.

Stimulation of the nicotinic acetylcholine receptor induces membrane depolarization by cation influx via the ion channel pore. We have therefore checked whether membrane depolarization induced by a high concentration of potassium chloride will cause the same effect on PYK2 tyrosine phosphorylation. Depolarization of PC12 cells with 75mM KCl induces rapid tyrosine phosphorylation of PYK2. The omission of calcium from the extracellular medium completely abolished PYK2 tyrosine phosphorylation, indicating that activation of PYK2 is due to calcium influx rather than membrane depolarization per

se. To further explore this possibility, we examined the effect of a calcium ionophore on PYK2 activation. PYK2 is phosphorylated on tyrosine residues following incubation with the calcium ionophore A23187. These results show
5 that elevation of intracellular calcium in response to a variety of stimuli causes tyrosine phosphorylation of PYK2.

Tyrosine phosphorylation of PYK2 in response to activation of a G protein coupled receptor was studied.
10 We analyzed the effect of bradykinin on the phosphorylation state of PYK2. Bradykinin induces rapid tyrosine phosphorylation of PYK2 in PC12 cells. By contrast to stimulation of PYK2 phosphorylation in response to carbachol treatment or to membrane
15 depolarization the effect of bradykinin was not influenced by the omission of extracellular calcium; bradykinin induced PYK2 phosphorylation in the absence of extracellular calcium or in the presence of EGTA.

Incubation of PC12 cells with phorbol myristate
20 acetate (PMA) induced tyrosine phosphorylation of PYK2, suggesting that tyrosine phosphorylation of PYK2 could also be mediated via protein kinase C (PKC) activation. To determine whether bradykinin-induced phosphorylation of PYK2 is mediated via PKC, the cells were treated with
25 bradykinin or PMA following down-regulation of PMA-sensitive PKC isozymes by prolonged treatment with PMA. Prolonged treatment with PMA completely abolished the effect of PMA, but had only a minor effect on bradykinin-stimulated tyrosine phosphorylation of PYK2. These
30 results suggest that tyrosine phosphorylation of PYK2 can be induced by PKC-independent and by PKC-dependents mechanisms.

Example 5: Phosphorylation of RAK

293 cells in 65mm plates were transiently
35 transfected either with the potassium channel-RAK-HA alone, or together with Fak, PYK2 or the PYK2-kinase

negative mutant (PKN). 12 hr following transfection the cells were grown in DMEM containing 0.3% fetal bovine serum for 24 hours. The cells were either stimulated with PMA (1.6 μ M) or with calcium ionophore A23187 (6 μ M) for 15 min at 37°C or left unstimulated. The cells were solubilized and the expression level of each protein was determined by western blot analysis. The Rak protein was immunoprecipitated by anti-HA antibodies and its phosphorylation on tyrosine residues was analyzed by western blot analysis utilizing anti-phosphotyrosine antibodies following immunoprecipitation of the proteins either with anti-PYK2 antibodies (for PYK2 and PKN) or with anti-Fak antibodies for (Fak).

The expression level of each protein (Rak, PYK2, PKN and Fak) and the tyrosine phosphorylation of Rak, PYK2, PKN and Fak were measured.

Only the kinase active PYK2 protein phosphorylated the potassium channel. No phosphorylation was observed with kinase negative PYK2 or with FAK.

Example 6: Tyrosine phosphorylation of PYK2 and Shc in response to activation of PC 12 cells by different stimuli.

PC12 cells were grown in DMEM containing 0.25% fetal bovine serum and 0.5% horse serum for 18 hours before stimulation. Following stimulation, the cells were washed with cold PBS and lysed in 0.8 ml lysis buffer (Lev et al., Mol. Cell. Biol. 13, 225-2234, 1993). PYK2 was immunoprecipitated by anti-PYK2 antibodies, the immunoprecipitates were resolved by 7.5% SDS-PAGE and immunoblotted either with anti-phosphotyrosine antibodies (RC20, transduction laboratories) or with anti-PYK2 antibodies. Antibodies against PYK2 were raised in rabbits.

Tyrosine phosphorylation of PYK2 in response to different stimuli was studied. Quiescent PC12 cells were stimulated at 37°C with carbachol (1mM, 20 sec),

bradykinin (1 μ M, 1min KCl (75mM, 3 min), PMA (1.6 μ M, 15min), A23187 (6 μ M, 15min) or left unstimulated (-). PYK2 was immunoprecipitated from cell lysates with anti-PYK2 antibodies, followed by SDS-PAGE and immunoblotting, with anti-phosphotyrosine or anti-PYK2 antibodies.

Tyrosine phosphorylation of Shc in response to bradykinin, carbachol, PMA and other stimuli was also measured. Quiescent PC12 cells were stimulated for 5 min at 37°C with bradykinin (1 μ M), carbachol (1mM), KCl (75mM), PMA (1.6 μ M), NGF(100ng/ml), or left unstimulated (-). The cells were also stimulated with carbachol (1mM) or potassium chloride (75mM) in the presence of 3mM EGTA. Stimulations with DMPP (100 μ M) or muscarine (1mM) were preformed under the same conditions. Time-course of carbachol induced tyrosine phosphorylation of Shc was performed by incubation of the cells with 1mM carbachol. The Shc proteins were immunoprecipitated with anti-Shc antibodies, the immunoprecipitates were resolved by SDS-PAGE (8%), transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine antibodies.

Example 7: Association of PYK2 with Grb2 and Sos 1 in intact cells.

In order to explore the possibility that calcium induced PYK2 activation is responsible for tyrosine phosphorylation of Shc and activation of the Ras/MAPK signaling pathway, we have examined the ability of PYK2 to recruit upstream regulatory elements of this signaling pathway, such as Shc and Grb2. Human embryonic 293 cells were transiently transfected with different combinations of expression vectors that direct the synthesis of PYK2, a kinase negative PYK2 mutant (PKN) and the adaptor protein Grb2. The results show that Grb2 is directly associated with wild type PYK2 but not with the kinase negative mutant. Experiments with GST-fusion protein of Grb2 indicate that the association between Grb2 and PYK2 is mediated via its SH2 domain. Inspection of PYK2

primary structure shows that tyr881 is followed by a LNV sequence which was shown to be a canonical binding site for the SH2 domain of Grb219.

We next examined the interaction of PYK2 with the
5 guanine nucleotide releasing factor SOS I. Human embryonic kidney 293 cells were transfected with expression vectors encoding SOS 1, PYK2 and PKIN and subjected to immunoprecipitation/immunoblotting analysis with anti Sos1 or anti-PYK2, antibodies, respectively.
10 Wild type PYK2 but not the kinase negative mutant (PKN) was co-immunoprecipitated with the with Sos1 protein. Hence, Grb2 is bound to Sos1 via its SH3 domains and to PYK2 via its SH2 domain leading to the recruitment of Sos by tyrosine phosphorylated PYK2.

15 Growth factor induced activation of receptor tyrosine kinases leads to a shift in the electrophoretic mobility of SOS protein. The mobility shift was shown to be due to phosphorylation by serine and threonine kinases which are dependent upon Ras activation including the MAP
20 kinase 11, 12, 20. SOS I protein from PYKI- transfected cells exhibits reduced electrophoretic mobility as compared to SOS I protein immunoprecipitated from P@ expressing cells. This experiment shows that PYK2 overexpression leads to the activation of the ser/thr
25 kinases responsible for the phosphorylation of SOS 1.

293 cells were transiently transfected with the full length cDNAs of PYK2, PKN Grb2 and hSos1-HA cloned into the mammalian expression vectors pRK5 downstream to the CMV promotor, using the calcium phosphate
30 precipitation method (Wigler et al., Cell 16, 777-785, 1979). Twelve hours after transfection, the cells were incubated in medium containing 0.2% fetal bovine serum for 24 hours. The cells were lysed, subjected to immunoprecipitation, resolved by SDS-PAGE (15% for Grb2
35 IPs, 7.5% for PYK2 IPs) and immunoblotted essentially as described (Lev et al., Mol. Cell Biol. 13, 2224-2234, 1993). For immunoblotting we used a mouse monoclonal

antibody against Grb2 (Transduction laboratories #GI6720). The kinase negative mutant of PYK2 was constructed as described. A mammalian expression vector encodes the hSos1-HA was constructed as described (Aronheim et al.,
5 Cell 78, 949-961, 1994).

Embryonic human kidney 293 cells were transiently transfected with different combinations of mammalian expression vectors that direct the synthesis of Grb2, PYK2 and a kinase negative PYK2 point mutant (PKN). The cells
10 were solubilized and immunoprecipitated with anti-Grb2, or anti-PYK2 antibodies. The immunocomplexes were washed, resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with either anti-PYK2, or anti-Grb2 antibodies. The expression level of Grb2 in each cell
15 line was determined by immunoblotting of total cell lysates with anti-Grb2 antibodies.

Embryonic human kidney 293 cells were transiently transfected with mammalian expression vectors encoding hSos1-HA, hSos1-HA together with PYK2 or hSos1-HA together
20 with PKN. hSos1 was immunoprecipitated with anti HA antibodies from each cell line, and the presence of PYK2 in the immunocomplexes was determined by immunoblotting with anti-PYK2 antibodies. Expression levels of hSos1, PYK2 and PKN were determined by immunoblot analysis of
25 total cell lysates, with anti-HA or anti PYK2 antibodies.

Example 8: PYK2 induces tyrosine phosphorylation of Shc and its association with Grb2.

Activated EGF receptor is able to recruit Grb2 directly and indirectly via tyrosine I 1, @ 1, 22. We
30 have therefore investigated whether PYK2 can induce phosphorylation of Shc tyrosine phosphorylation of Shc and its association with Grb2. Shc proteins were immunoprecipitated with anti Shc antibodies from Shc, from Shc and PYK2, or from Shc and PKN expressing cells. The
35 samples were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine

or anti-Grb2 antibodies. Dramatic tyrosine phosphorylation of Shc in cells that overexpress PYK2. Moreover, several phosphotyrosine containing proteins were found in Shc immunoprecipitates from PYK2 overexpressing
5 cells. Similar results were observed in cells expressing endogenous Shc proteins that were transfected with PYK2 CDNA and subject to immunoprecipitation analysis with anti Shc antibodies. Immunoblot analysis with Grb2 antibodies of Shc immunoprecipitates indicated that Grb2 associates
10 with tyrosine phosphorylated Shc in PYK2 overexpressing cells. We therefore conclude that tyrosine phosphorylated PYK2 can directly and indirectly recruit Grb2 via tyrosine phosphorylation of Shc revealing at least two alternative routes for PYK2 induced activation of the Ras signaling
15 pathway.

Tyrosine phosphorylation of Shc in cells that coexpress PYK2 was standard. Cells that express Shc alone or coexpress Shc together with either PYK2, or PKN were lysed and subjected to immunoprecipitation with anti-Shc
20 antibodies or pre-immune serum (P.I.). The immunocomplexes were washed, run on an SDS gel and immunoblotted with anti-phosphotyrosine antibodies. Shc proteins (46, 52 and 66kDa) were identified.

PYK2 induces association of Shc with Grb2. Shc
25 proteins were immunoprecipitated from each cell line using anti-Shc antibodies. As a control, the lysates of cells that coexpress PYK2 and Shc were subject to immunoprecipitation with pre-immune serum (P.I.). The presence of Grb2 in the immunocomplexes was determined by
30 immunoblotting with anti-Grb2 antibodies.

The expression level of of PYK2, PKN and Shc in each cell line was determined by immunoblot analysis of total cell lysates with specific antibodies as indicated.

Example 9: Activation of MAP kinase in PC12 cells by bradykinin, carbachol and other stimuli.

The experiments presented so far show that the same stimuli that induce activation of PYK2 induce
5 tyrosine phosphorylation of Shc. We next examined the ability of these agents to induce the activation of kinases in PC12 cells. Quiescent PC12 cells were incubated with a variety of stimuli. Lysates from stimulated cells were subjected to immunoprecipitation
10 with anti-MAP kinase antibodies followed by immunoblotting, with phosphotyrosine antibodies. Myelin basic protein (MBP) was utilized as a substrate to determine MAP kinase activation. The addition of various ligands to PC12 cells induced a similar profile of both
15 tyrosine phosphorylation and activation of MAP kinase in these cells.

Since activation of MAP kinase was observed in response to stimuli that induce PYK2 phosphorylation, we examined the possibility whether PYK2 overexpression can
20 induce MAP kinase activation. Human embryonic kidney 293 cells were transiently transfected with increasing concentrations of mammalian expression vector that directs the synthesis of PYK2. The cells were grown for 24 hours in the presence of 0.2% serum, MAPK 1,2 proteins were
25 immunoprecipitated, washed and subjected to MBP phosphorylation assay. The results presented in figure 4b show that PYK2 overexpression induced MBP phosphorylation in a concentration dependent manner.

Quantitation of these results shows that MAP
30 kinase activity was approximately three fold higher in cells that expressed the highest level of PYK2 as compared to mock transfected cells.

293 cells were transiently transfected with mammalian expression vectors for Shc alone, Shc together
35 with PYK2, or Shc together with PKN. PC12 cells were starved for 18 hours as described. The cells were stimulated for 5 min at 37 C with the indicated stimuli,

lysed and subjected to immunoprecipitation with antiMAPK 1,2 antibodies (Santa Cruz Biotechnology, #c-14 and #c-16). The immunoprecipitates were washed twice with lysis buffer (Lev et al., Mol. Cell. Biol. 13, 2224-2234, 1993) and once with Tris-buffer containing 10mM Tris-HCl pH 7.2, 100mM NaCl, 1mM Na-vanadate and 5mM benzamide. The immunocomplexes were resuspended in 40µl of MAP kinase-buffer containing 30mM Tris-HCl pH 8, 20mM MgCl₂, 2mM MnCl₂, 15µg, MBP, 10µM ATP and 5µCi γ -[³²P]ATP (Amersham). The samples were incubated for 30 min at 30°C and the reactions were stopped by the addition of SDS-sample buffer. The samples were resolved on 15% SDS-PAGE and analysed by autoradiography. Human embryonic kidney 293 cells were transiently transfected with increasing concentration of pRK5-PYK2 DNA (0.5-µg). Twelve hours after transfection the cells were (grown in medium containing 0.2% serum for 24 hours. The cells were lysed, immunoprecipitated with MAPK 1,2 antibodies and subjected to MBP phosphorylation assay as describe above.

Quiescent PC12 cells were stimulated for 5 min at 37°C with bradykinin (1µM), carbachol (1mM), KCl (75mM), PMA (1.6µM), NGF (100mg/ml), or left unstimulated (-). The cells were lysed and MAPK 1,2 were immunoprecipitated with specific antibodies. The immunocomplexes were washed and either resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine antibodies, or subjected to a standard myelin basic protein (MBP) phosphorylation assay.

Activation of MAP kinase by overexpression of PYK2. Human embryonic kidney 293) cells were transiently transfected with increasing concentrations of a mammalian expression vector that directs the synthesis of PYK2. MAPK 1,2 proteins were immunoprecipitated from each cell line, the immunocomplexes were washed and subjected to MBP phosphorylation assay. Quantitation of MAP kinase activity for each cell line was determined by phosphorimager and Imager software (Molecular Dynamics,

Incorporated). MAPK activity in transfected cells is compared to activity detected in control mock transfected cells.

Example 10: Bradykinin stimulation of PC12 cells induces tyrosine phosphorylation of PYK2.

Ligand stimulation, immunoprecipitations and immunoblotting were performed. Chronic treatment with PMA was performed by incubation of the cells with 100nM PMA for 12 hours at 37°C.

Time course of bradykinin induces tyrosine phosphorylation of PYK2. Quiescent PC12 cells were incubated at 37°C with 1 μ M bradykinin for indicated periods of time. PYK2 was immunoprecipitated from untreated (-) or treated cells, the immunocomplexes were washed, resolved by SDS-PAGE, transferred to nitrocellulose, and probed either with anti-phosphotyrosine or anti-PYK2 antibodies.

Quiescent PC12 cells were incubated with either 1 μ M bradykinin (1 min at 37°C) or with PMA (1.6 μ M, 15 min at 37°C) in the presence or absence of CaCl or EGTA (3 μ M) as indicated. In some cases the cells were pretreated with 100nM PMA for 12 h. PYK2 was immunoprecipitated from stimulated or unstimulated cells (-) and analysed by immunoblot analysis with either anti-phosphotyrosine or anti-PYK2 antibodies.

Example 11: Stimulation of Kv1.2 potassium channel tyrosine phosphorylation in response to PYK2 activation.

We examined the possibility whether PYK2 can tyrosine phosphorylate the Kv1.2 channel and regulate its function. In order to test this possibility, we expressed in 293 cells the Kv1.2 protein, Kv1.2 together with PYK2, and as a control Kv1.2 with a kinase negative PYK2 mutant (PKN) or with the protein tyrosine kinase Fak. The cells were grown for 24 hours in medium containing 0.2% serum

and then stimulated with PMA(1.6 μ M), calcium ionophore (6 μ M), or left unstimulated.

Immunoblotting analysis with phosphotyrosine antibodies following immunoprecipitation of PYK2, PKN and
5 Fak by specific antibodies. PYK2 and Fak were phosphorylated on tyrosine even in unstimulated cells, and treatment with PMA induced tyrosine phosphorylation while treatment with calcium ionophore induced a weaker response. The level of expression of the kinase negative
10 mutant of PYK2 (PKN) was similar to the expression of wild type PYK2 or FAK. Nevertheless, as expected, PKN was not found to be phosphorylated on tyrosine residues. We next analyzed the tyrosine phosphorylation of Kv1.2 channel in each cell line.

15 We have added to the cDNA expression construct of Kv1.2 an HA tag, and determined the level of Kv1.2 expression by immunoblot analysis with anti-HA antibodies. A similar amount of Kv1.2 protein was expressed in the transfected cell lines. The Kv1.2 protein was
20 immunoprecipitated from unstimulated cells, as well as from, PMA or calcium ionophore stimulated cells. The immunoprecipitates were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibodies. Phosphorylation of Kv1.2 on tyrosine residues was observed
25 only in cells coexpressing, PYK2. Moreover, tyrosine phosphorylation of Kv1.2 was enhanced by PMA or calcium ionophore treatments indicating that activation of PYK2 is required for PYK2 induced tyrosine phosphorylation of the potassium channel.

30 Embryonic human kidney 293 cells were transiently transfected with different combinations of mammalian expression vectors which direct the synthesis of Kv1.2-HA, PYK2, a kinase negative PYK2 (PKN) or the protein tyrosine kinase Fak. The cells were grown for 24 h in the presence
35 of 0.2% serum and then either stimulated with PMA (1.6 μ M, 10 min at 37°C), the calcium ionophore A23187 (6 μ M, 10 min at 37°C) or left unstimulated (-).

Tyrosine phosphorylation of each protein was analysed following immunoprecipitation and immunoblotting with anti-phosphotyrosine antibodies. The expression of each protein was determined by immunoblot analysis of
5 total cell lysates from each transfection with anti-PYK2, anti- HA or anti-Fak antibodies.

Tyrosine phosphorylation of Kv1.2 was analysed by immunoprecipitation of Kv1.2-HA protein from each cell line with anti-HA antibodies, followed by immunoblot
10 analysis with anti-phosphotyrosine antibodies. 293 cells were transfected by the calcium phosphate technique as described (Wigler et al., Cell 16, 777-785, 1979). The influenza virus hemagglutinin peptide (YPYDVPDYAS) [SEQ. ID NO 22] tag was added to the C-terminal end of the Kv1.2
15 cDNA utilizing the following oligonucleotide primers in the PCR; '5GCCAGCAGGCCATGTCACTGG-3' [SEQ. ID NO 23] and '5CGGAATTCTTACGATGCGTAGTCAGGGACATCGTATGGGTAGACATCAGTTAAC ATT TTG-'3 [SEQ. ID NO 24]. The PCR product was digested with BAII and EcoRI and used to substitute the
20 corresponding fragment at the C-terminal end of the Kv1.2 cDNA. The Kv1.2-HA cDNA was subcloned into pRK5 downstream the CMV promoter.

A kinase negative mutant of PYK2 (PKN) was constructed by replacing Lys475 with an Ala residue by
25 utilizing a site directed mutagenesis Kit (Clontech). The oligonucleotide sequence was designed to create a new NruI restriction site. The nucleotide sequence of the mutant was confirmed by DNA sequencing. The oligonucleotide sequence that used for mutagenesis is: '5-
30 CAATGTAGCTGTGCGACCTGCAAGAAAGAC-3' [SEQ. ID NO 25] (NruI site - bold, Lys-AAC substituted to Ala-GCG underline). The full length cDNAs of PYK2, PKN and Fak were subcloned into the mammalian expression vectors pRK5 downstream to the CMV promoter.

Example 12: Suppression of potassium channel action in frog oocytes by PYK2 expression and PMA treatment.

In vitro capped RNA transcripts of Kv1.2, PYK2 and PKN were synthesized from linearized plasmids DNA templates utilizing the mMESSAGE mMACHINE kit (Ambion), following the supplier's protocols. The products of the transcription reaction (cRNAs) were diluted in RNase-free water and stored at -70°C. Expression of the RNAs was done by injection of 50nl of RNA into defolliculated stage V and VI oocytes from *Xenopus laevis* (Iverson et al., J. Neurosc. 10, 2903-2916, 1990). The injected oocytes were incubated for 2-3 days at 20°C in L15 solution (1:2 dilution of Gibco's Leibovitz L15 medium in H2O, with 50U/ml nystatin, 0.1mg/ml gentamycin, 30mM HEPES buffer, pH 7.3-7.4, filtered through a 0.45mm membrane). Electrophysiological Recording and analysis. Ionic currents were recorded with a two microelectrode voltage-clamp as described (Iverson et al., *supra*). The current were low-pass filtered KHz using an 8-pole Bessel filter and stored in a 80286 microcomputer using the pClamp acquisition system (Axon Instruments). The data was analyzed with the clamp fit pro-rams of the pClamp system (Axon Instruments). All recording were performed at room temperature (20-23°C). The recording chamber was continually perfused with recording solution. To avoid contamination of the oocyte by Ca²⁺-activated Cl⁻ currents low Cl⁻ recording solution was used (96mM Na⁺, glutamate, 2mM K⁺ glutamate, 0.5mM CaCl₂, 5mM MgCl₂, 5mM HEPES buffer). The K⁺ currents were elicited in depolarizing steps from -100 to +40 mV in 10 mV increments every 15 seconds.

Kv1.2 currents from oocytes microinjected with either Kv1.2 mRNA, Kv1.2 and PYK2 mRNAs, or Kv1.2 and a kinase negative mutant of PYK2 mRNAs (PKN). Currents were elicited in response to depolarizing steps from -100 to +30 mV increments from a holding potential of -110 mV. Representative traces of Kv1.2 channels before and after

bath application of 100 nM PMA at the annotated time (8 and 20 minutes) in the same cell.

Suppression of Kv1.2 currents in response to PMA is blocked by a kinase negative PYK2 mutant (PKN).
5 Inhibition of Kv1.2 currents in an oocyte microinjected with Kv1.2 mRNA before and 25 minutes after treatment with PMA at 50 nM or 100 nM concentration. Recordings from an oocyte expression Kv1.2 and PYK2 or Kv1.2 and a kinase negative mutant of PYK2 under the same conditions as
10 described above. The same protocol was utilized in both experiments.

We asked whether stimulation of PYK2 can suppress Kv1.2 currents. We explored the effect of PYK2 expression, on currents exhibited by Kv1.2 expression in
15 *Xenopus* oocytes. Stage V oocytes were microinjected either with Kv1.2 transcripts or with Kv1.2 together with PYK2 or PKN mRNAs. Following two to three days of incubation at 20°C, macroscopic currents exhibited by the oocytes were recorded with a two microelectrode voltage
20 clamp as described (Iverson et al., J. Neurosci. 10, 2903-2916, 1990). Outward rectifier currents were recorded upon membrane depolarization above -40mV, indicating that a functional Kv1.2 channel is expressed in the oocytes. The expression of Kv1.2, PYK2 and PKN in the frog oocytes
25 was confirmed by immunoblot analysis with anti-HA or anti-PYK2 antibodies.

We have examined the effect of PYK2 expression on Kv1.2 currents in oocytes in the absence or presence of PMA. We also examined the effect of the kinase negative
30 mutant PKN on PMA induced suppression of Kv 1.2 currents mediated by the endogenous protein tyrosine kinase. Treatment of oocytes with PMA caused inhibition of Kv1.2 currents. As previously shown, the inhibition of the currents developed gradually after application of PMA
35 reaching 80-90% inhibition after 20 min incubation (Huang et al., Cell 75, 1145-1156, 1993). Moreover, the rate of channel blockade was found to be dependent upon the

concentration of PMA applied. Coexpression of PYK2 resulted in acceleration of Kv1.2 currents inhibition. Significant acceleration of current inhibition was observed at every concentration of PMA tested. For
5 example, 8 min after the addition of 100 nM PMA, 25% inhibition of outward current was observed in oocytes expressing Kv1.2 alone as compared to 95% inhibition observed in oocytes coexpressing Kv1.2 and PYK2 proteins.

Current inhibition by PMA treatment in the
10 absence or presence of PYK2 expression did not result in changes in both the kinetics or voltage dependence of the remaining currents. Coexpression of Kv1.2 together with the kinase negative mutant of PYK2 (PKN) led to nearly complete inhibition of PMA induced potassium channel
15 blockage. It is possible that the endogenous protein tyrosine kinase activated by PMA that is responsible for suppression of Kv1.2 currents in oocytes represents the xenopus homologue of PYK2 or a closely related protein tyrosine kinase that can be affected by a dominant
20 interfering mutant of PYK2.

Other embodiments are within the following claims.

	CELL LINE	CHECKED BY	LEVEL OF EXPRESSION	COMMENT
	NIH3T3	IP/IB	-	High expression of Fak (IP/IB)
	L cells	IP/IB	-	-
5	Jurak (T-lymphoblastic cells)	IP/IB	+	very high expression of Fak (IP/IB)
	KG-1 (human myeloblast/promyelocyte)	IP/IB	+++	-
10	K562 (human erythroleukemia)	IP/IB	++	-
	CHRF (premegakaryocyte, human)	IP/IB	+++	After differentiation with TPA for 3 days, mobility shift of PYK2
15	L8057 (premegakaryocyte, mouse)	IP/IB	++	After differentiation with TPA for 3 days, mobility shift of PYK2
	T47D (human breast carcinoma)	IP/IB, PCR	++	PCR gave higher expression as compared to the IP/IB
20	GH3 (Pituitary tumor, rat)	IP/IB	++	-
	PC12	IP/IB, PCR, Northern	+++	No change of PYK2 expression level or mobility after 36hr treatment with NGF, No expression of Fak (IP/IB)
	XC (Sarcoma, rat)	IP/IB	+++	PYK2 is phosphorylated on tyrosine
25	HEL (human erythroleukemia/myeloid)	IP/IB	++	-
	HL-60 (human promyelocytic leukemia)	IP/IB	++++	-
30	NG108-15 (neuroblastoma-glioma hybrid)	IP/IB	+	-

TABLE 1

122

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(ii) TITLE OF INVENTION: PYK2 RELATED PRODUCTS AND METHODS

5 (iii) NUMBER OF SEQUENCES: 24

(iv) CORRESPONDENCE ADDRESS:

10 (A) ADDRESSEE: Lyon & Lyon
(B) STREET: 633 West Fifth Street
(C) CITY: Los Angeles
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 90071

(v) COMPUTER READABLE FORM:

15 (A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb storage
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: IBM P.C. DOS (Version 5.0)
(D) SOFTWARE: WordPerfect (Version 5.1)

(vi) CURRENT APPLICATION DATA:

20 (A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:

25 (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Warburg, Richard J.
(B) REGISTRATION NUMBER: 32,327
(C) REFERENCE/DOCKET NUMBER: 211/121

(ix) TELECOMMUNICATION INFORMATION:

30 (A) TELEPHONE: (213) 489-1600
(B) TELEFAX: (213) 955-0440
(C) TELEX: 67-3510

35 (2) INFORMATION FOR SEQUENCE ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

40 (A) LENGTH: 1009
(B) TYPE: amino acid
(C) STRANDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

123

Met Ser Gly Val Ser Glu Pro Leu Ser Arg Val Lys Leu Gly Thr Leu
 1 5 10 15
 5 Arg Arg Pro Glu Gly Pro Ala Glu Pro Met Val Val Val Pro Val Asp
 20 25 30
 Val Glu Lys Glu Asp Val Arg Ile Leu Lys Val Cys Phe Tyr Ser Asn
 35 40 45
 10 Ser Phe Asn Pro Gly Lys Asn Phe Lys Leu Val Lys Cys Thr Val Gln
 50 55 60
 Thr Glu Ile Arg Glu Ile Ile Thr Ser Ile Leu Leu Ser Gly Arg Ile
 65 70 75 80
 Gly Pro Asn Ile Arg Leu Ala Glu Cys Tyr Gly Leu Arg Leu Lys His
 85 90 95
 15 Met Lys Ser Asp Glu Ile His Trp Leu His Pro Gln Met Thr Val Gly
 100 105 110
 Glu Val Gln Asp Lys Tyr Glu Cys Leu His Val Glu Ala Glu Trp Arg
 115 120 125
 20 Tyr Asp Leu Gln Ile Arg Tyr Leu Pro Glu Asp Phe Met Glu Ser Leu
 130 135 140
 Lys Glu Asp Arg Thr Thr Leu Leu Tyr Phe Tyr Gln Gln Leu Arg Asn
 145 150 155 160
 Asp Tyr Met Gln Arg Tyr Ala Ser Lys Val Ser Glu Gly Met Ala Leu
 165 170 175
 25 Gln Leu Gly Cys Leu Glu Leu Arg Arg Phe Phe Lys Asp Met Pro His
 180 185 190
 Asn Ala Leu Asp Lys Lys Ser Asn Phe Glu Leu Leu Glu Lys Glu Val
 195 200 205
 30 Gly Leu Asp Leu Phe Phe Pro Lys Gln Met Gln Glu Asn Leu Lys Pro
 210 215 220
 Lys Gln Phe Arg Lys Met Ile Gln Gln Thr Phe Gln Gln Tyr Ala Ser
 225 230 235 240
 Leu Arg Glu Glu Glu Cys Val Met Lys Phe Phe Asn Thr Leu Ala Gly
 245 250 255
 35 Phe Ala Asn Ile Asp Gln Glu Thr Tyr Arg Cys Glu Leu Ile Gln Gly
 260 265 270
 Trp Asn Ile Thr Val Asp Leu Val Ile Gly Pro Lys Gly Ile Arg Gln
 275 280 285
 40 Leu Thr Ser Gln Asp Ala Lys Pro Thr Cys Leu Ala Glu Phe Lys Gln
 290 295 300
 Ile Arg Ser Ile Arg Cys Leu Pro Leu Glu Glu Gly Gln Ala Val Leu
 305 310 315 320
 Gln Leu Gly Ile Glu Gly Ala Pro Gln Ala Leu Ser Ile Lys Thr Ser
 325 330 335
 45 Ser Leu Ala Glu Ala Glu Asn Met Ala Asp Leu Ile Asp Gly Tyr Cys
 340 345 350

124

	Arg	Leu	Gln	Gly	Glu	His	Gln	Gly	Ser	Leu	Ile	Ile	His	Pro	Arg	Lys	
			355					360					365				
	Asp	Gly	Glu	Lys	Arg	Asn	Ser	Leu	Pro	Gln	Ile	Pro	Met	Leu	Asn	Leu	
		370					375					380					
5	Glu	Ala	Arg	Arg	Ser	His	Leu	Ser	Glu	Ser	Cys	Ser	Ile	Glu	Ser	Asp	
		385				390					395					400	
	Ile	Tyr	Ala	Glu	Ile	Pro	Asp	Glu	Thr	Leu	Arg	Arg	Pro	Gly	Gly	Pro	
					405					410					415		
10	Gln	Tyr	Gly	Ile	Ala	Arg	Glu	Asp	Val	Val	Leu	Asn	Arg	Ile	Leu	Gly	
					420					425					430		
	Glu	Gly	Phe	Phe	Gly	Glu	Val	Tyr	Glu	Gly	Val	Tyr	Thr	Asn	His	Lys	
				435					440					445			
	Gly	Glu	Lys	Ile	Asn	Val	Ala	Val	Lys	Thr	Cys	Lys	Lys	Asp	Cys	Thr	
			450					455					460				
15	Leu	Asp	Asn	Lys	Glu	Lys	Phe	Met	Ser	Glu	Ala	Val	Ile	Met	Lys	Asn	
		465					470					475					
	Leu	Asp	His	Pro	His	Ile	Val	Lys	Leu	Ile	Gly	Ile	Ile	Glu	Glu	Glu	
		480				485					490					495	
20	Pro	Thr	Trp	Ile	Ile	Met	Glu	Leu	Tyr	Pro	Tyr	Gly	Glu	Leu	Gly	His	
					500					505					510		
	Tyr	Leu	Glu	Arg	Asn	Lys	Asn	Ser	Leu	Lys	Val	Leu	Thr	Leu	Val	Leu	
				515					520					525			
	Tyr	Ser	Leu	Gln	Ile	Cys	Lys	Ala	Met	Ala	Tyr	Leu	Glu	Ser	Ile	Asn	
			530					535					540				
25	Cys	Val	His	Arg	Asp	Ile	Ala	Val	Arg	Asn	Ile	Leu	Val	Ala	Ser	Pro	
		545					550					555					
	Glu	Cys	Val	Lys	Leu	Gly	Asp	Phe	Gly	Leu	Ser	Arg	Tyr	Ile	Glu	Asp	
		560				565					570					575	
30	Glu	Asp	Tyr	Tyr	Lys	Ala	Ser	Val	Thr	Arg	Leu	Pro	Ile	Lys	Trp	Met	
					580					585					590		
	Ser	Pro	Glu	Ser	Ile	Asn	Phe	Arg	Arg	Phe	Thr	Thr	Ala	Ser	Asp	Val	
				595					600					605			
	Trp	Met	Phe	Ala	Val	Cys	Met	Trp	Glu	Ile	Leu	Ser	Phe	Gly	Lys	Gln	
				610					615					620			
35	Pro	Phe	Phe	Trp	Leu	Glu	Asn	Lys	Asp	Val	Ile	Gly	Val	Leu	Glu	Lys	
			625					630					635				
	Gly	Asp	Arg	Leu	Pro	Lys	Pro	Asp	Leu	Cys	Pro	Pro	Val	Leu	Tyr	Thr	
		640					645					650					
40	Leu	Met	Thr	Arg	Cys	Trp	Asp	Tyr	Asp	Pro	Ser	Asp	Arg	Pro	Arg	Phe	
		655				660					665					670	
	Thr	Glu	Leu	Val	Cys	Ser	Leu	Ser	Asp	Val	Tyr	Gln	Met	Glu	Lys	Asp	
					675					680					685		
	Ile	Ala	Met	Glu	Gln	Glu	Arg	Asn	Ala	Arg	Tyr	Arg	Thr	Pro	Lys	Ile	
					690				695					700			

125

Leu Glu Pro Thr Ala Phe Gln Glu Pro Pro Pro Lys Pro Ser Arg Pro
 705 710 715
 Lys Tyr Arg Pro Pro Pro Gln Thr Asn Leu Leu Ala Pro Lys Leu Gln
 720 725 730
 5 Phe Gln Val Pro Glu Gly Leu Cys Ala Ser Ser Pro Thr Leu Thr Ser
 735 740 745 750
 Pro Met Glu Tyr Pro Ser Pro Val Asn Ser Leu His Thr Pro Pro Leu
 755 760 765
 10 His Arg His Asn Val Phe Lys Arg His Ser Met Arg Glu Glu Asp Phe
 770 775 780
 Ile Gln Pro Ser Ser Arg Glu Glu Ala Gln Gln Leu Trp Glu Ala Glu
 785 790 795
 Lys Val Lys Met Arg Gln Ile Leu Asp Lys Gln Gln Lys Gln Met Val
 800 805 810
 15 Glu Asp Tyr Gln Trp Leu Arg Gln Glu Glu Lys Ser Leu Asp Pro Met
 815 820 825 830
 Val Tyr Met Asn Asp Lys Ser Pro Leu Thr Pro Glu Lys Glu Val Gly
 835 840 845
 20 Tyr Leu Glu Phe Thr Gly Pro Pro Gln Lys Pro Pro Arg Leu Gly Ala
 850 855 860
 Gln Ser Ile Gln Pro Thr Ala Asn Leu Asp Arg Thr Asp Asp Leu Val
 865 870 875
 Tyr Leu Asn Val Met Glu Leu Val Arg Ala Val Leu Glu Leu Lys Asn
 880 885 890
 25 Glu Leu Cys Gln Leu Pro Pro Glu Gly Tyr Val Val Val Val Lys Asn
 895 900 905 910
 Val Gly Leu Thr Leu Arg Lys Leu Ile Gly Ser Val Asp Asp Leu Leu
 915 920 925
 30 Pro Ser Leu Pro Ser Ser Ser Arg Thr Glu Ile Glu Gly Thr Gln Lys
 930 935 940
 Leu Leu Asn Lys Asp Leu Ala Glu Leu Ile Asn Lys Met Arg Leu Ala
 945 950 955
 Gln Gln Asn Ala Val Thr Ser Leu Ser Glu Glu Cys Lys Arg Gln Met
 960 965 970
 35 Leu Thr Ala Ser His Thr Leu Ala Val Asp Ala Lys Asn Leu Leu Asp
 975 980 985 990
 Ala Val Asp Gln Ala Lys Val Leu Ala Asn Leu Ala His Pro Pro Ala
 995 1000 1005
 Glu

40 (2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3416
 (B) TYPE: nucleic acid

126

(C) STRANDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

5	CGGTACAGGT AAGTCGGCCG GGCAGGTAGG GGTGCCCCGAG GAGTAGTCGC TGGAGTCCGC	60
	GCCTCCCTGG GACTGCAATG TGCCGGTCTT AGCTGCTGCC TGAGAGGATG TCTGGGGTGT	120
	CCGAGCCCCCT GAGCCGAGTA AAGTTGGGCA CATTACGCCG GCCTGAAGGC CCTGCAGAGC	180
	CCATGGTGGT GGTACCAGTA GATGTGGAAA AGGAGGACGT GCGTATCCTC AAGGTCTGCT	240
	TCTATAGCAA CAGCTTCAAT CCTGGGAAGA ACTTCAAACCT GGTCAAATGC ACTGTCCAGA	300
10	CGGAGATCCG GGAGATCATC ACCTCCATCC TGCTGAGCGG GCGGATCGGG CCCAACATCC	360
	GGTTGGCTGA GTGCTATGGG CTGAGGCTGA AGCACATGAA GTCCGATGAG ATCCACTGGC	420
	TGCACCCACA GATGACGGTG GGTGAGGTGC AGGACAAGTA TGAGTGCTG CACGTGGAAG	480
	CCGAGTGGAG GTATGACCTT CAAATCCGCT ACTTGCCAGA AGACTTCATG GAGAGCCTGA	540
15	AGGAGGACAG GACCACGCTG CTCTATTTT ACCAACAGCT CCGGAACGAC TACATGCAGC	600
	GCTACGCCAG CAAGGTCAGC GAGGGCATGG CCCTGCAGCT GGGCTGCCTG GAGCTCAGGC	660
	GGTTCTTCAA GGATATGCC CACAATGCAC TTGACAAGAA GTCCAACTTC GAGCTCCTAG	720
	AAAAGGAAGT GGGGCTGGAC TTGTTTTTCC CAAAGCAGAT GCAGGAGAAC TTAAAGCCCA	780
	AACAGTTCGG GAAGATGATC CAGCAGACCT TCCAGCAGTA CGCCTCGCTC AGGGAGGAGG	840
20	AGTGCCTCAT GAAGTTCTTC AACACTCTCG CCGGCTTCGC CAACATCGAC CAGGAGACCT	900
	ACCCTGTGTA ACTCATTCAA GGATGGAACA TTACTGTGGA CCTGGTCATT GGCCTAAAG	960
	GGATCCGCCA GCTGACTAGT CAGGACGCAA AGCCACCTG CCTGGCCGAG TTCAGCAGA	1020
	TCAGGTCCAT CAGGTGCCTC CCGCTGGAGG AGGGCCAGGC AGTACTTCAG CTGGGCATTG	1080
	AAGGTGCCCC CCAGGCCTTG TCCATCAAAA CCTCATCCCT AGCAGAGGCT GAGAACATGG	1140
25	CTGACCTCAT AGACGGCTAC TGCCGGCTGC AGGGTGAGCA CCAAGGCTCT CTCATCATCC	1200
	ATCCTAGGAA AGATGGTGAG AAGCGGAACA GCCTGCCCCA GATCCCCATG CTAAACCTGG	1260
	AGGCCCCGGG GTCCACCTC TCAGAGAGCT GCAGCATAGA GTCAGACATC TACGCAGAGA	1320
	TTCCCCAGCA AACCTGCGA AGGCCCCGAG GTCCACAGTA TGGCATTGCC CGTGAAGATG	1380
	TGGTCCTGAA TCGTATTCTT GGGGAAGGCT TTTTGGGGA GGTCTATGAA GGTGTCTACA	1440
30	CAAATCACAA AGGGGAGAAA ATCAATGTAG CTGTCAAGAC CTGCAAGAAA GACTGCACTC	1500
	TGGACAACAA GGAGAAGTTC ATGAGCGAGG CAGTGATCAT GAAGAACCTC GACCACCCGC	1560
	ACATCGTGAA GCTGATCGGC ATCATTGAAG AGGAGCCAC CTGGATCATC ATGGAATTGT	1620
	ATCCCTATGG GGAGCTGGGC CACTACCTGG AGCGGAACAA GAACTCCCTG AAGGTGCTCA	1680
	CCCTCGTGCT GTACTACTG CAGATATGCA AAGCCATGGC CTACCTGGAG AGCATCAACT	1740
35	GCGTGCACAG GGACATTGCT GTCCGGAACA TCCTGGTGGC CTCCCCTGAG TGTGTGAAGC	1800
	TGGGGGACTT TGGTCTTTCC CGGTACATTG AGGACGAGGA CTATTACAAA GCCTCTGTGA	1860
	CTCGTCTCCC CATCAAATGG ATGTCCCCAG AGTCCATTAA CTTCCGACGC TTCACGACAG	1920
	CCAGTGACGT CTGGATGTTT GCCGTGTGCA TGTGGGAGAT CCTGAGCTTT GGAAGCAGC	1980

127

CCTTCTTCTG GCTGGAGAAC AAGGATGTCA TCGGGGTGCT GGAGAAAGGA GACCGGCTGC 2040
CCAAGCCTGA TCTCTGTCCA CCGGTCCTTT ATACCCTCAT GACCCGCTGC TGGGACTACG 2100
ACCCCACTGA CCGGCCCCGC TTCACCGAGC TGGTGTGCAG CCTCAGTGAC GTTTATCAGA 2160
TGGAGAAGGA CATTGCCATG GAGCAAGAGA GGAATGCTCG CTACCGAACC CCCAAAATCT 2220
5 TGGAGCCAC AGCCTTCCAG GAACCCAC CCAAGCCCAG CCGACCTAAG TACAGACCCC 2280
CTCCGCAAAC CAACCTCCTG GCTCCAAAGC TGCAATTCCA GGTTCTGAG GGTCTGTGTG 2340
CCAGCTCTCC TACGCTCACC AGCCCTATGG AGTATCCATC TCCCGTTAAC TCACTGCACA 2400
CCCCACCTCT CCACCGGCAC AATGTCTTCA AACGCCACAG CATGCGGGAG GAGGACTTCA 2460
TCCAACCCAG CAGCCGAGAA GAGGCCACG AGCTGTGGGA GGCTGAAAAG GTCAAAATGC 2520
10 GGCAAATCCT GGACAAACAG CAGAAGCAGA TGGTGGAGGA CTACCACTGG CTCAGGCAGG 2580
AGGAGAAGTC CCTGGACCCC ATGGTTTATA TGAATGATAA GTCCCCATTG ACGCCAGAGA 2640
AGGAGGTCGG CTACCTGGAG TTCACAGGC CCCACAGAA GCCCCGAGG CTGGGCGCAC 2700
AGTCCATCCA GCCCACAGCT AACCTGGACC GGACCGATGA CCTGGGTGAC CTCAATGTCA 2760
TGGAGCTGGT GCGGGCCGTG CTGGAGCTCA AGAATGAGCT CTGTCAGCTG CCCCCGAGG 2820
15 GCTACGTGGT GGTGGTGAAG AATGTGGGC TGACCCTGCG GAAGCTCATC GGGAGCGTGG 2880
ATGATCTCCT GCCTTCCTTG CCGTCATCTT CACGACAGA GATCGAGGGC ACCCAGAAAC 2940
TGCTCAACAA AGACCTGGCA GAGCTCATCA ACAAGATGCG GCTGGCGCAG CAGAACGCCG 3000
TGACCTCCCT GAGTGAGGAG TGCAAGAGGC AGATGCTGAC GGCTTCACAC ACCCTGGCTG 3060
TGGACGCCAA GAACCTGCTC GACGCTGTGG ACCAGGCCAA GGTTCGGCC AATCTGGCCC 3120
20 ACCCACTGC AGAGTGACGG AGGGTGGGG CCACCTGCCT GCGTCTCCG CCCCTGCCTG 3180
CCATGTACCT CCCCTGCCTT GCTGTTGGTC ATGTGGGTCT TCCAGGGAGA AGGCCAAGGG 3240
GAGTCACCTT CCCTTGCCAC TTTGCACGAC GCCCTCTCCC CACCCCTACC CCTGGCTGTA 3300
CTGCTCAGGC TGCAGCTGGA CAGAGGGGAC TCTGGGCTAT GGACACAGGG TGACGGTGAC 3360
25 AAAGATGGCT CAGAGGGGGA CTGCTGCTGC CTGGCCACTG CTCCCTAAGC CAGCCT 3416

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Ile His Arg Asp Leu Ala Ala Arg Asn
5 10

40 (2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

128

(A) LENGTH: 8
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Trp Met Phe Gly Val Thr Leu Trp
5

10 (2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 30
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

20 CGGGATCCTC ATCATCCATC CTAGGAAAGA 30

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 30
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30 (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CGGGAATTCG TCGTAGTCCC AGCAGCGGGT 30

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Tyr Pro Tyr Asp Val Pro Asp Tyr Ala Ser
5 10

45 (2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11
(B) TYPE: nucleic acid

129

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

5 CACAATGTCT TCAAACGCCA C 11

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 63
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GGCTCTAGAT CACGATGCGT AGTCAGGGAC ATCGTATGGG TACTCTGCAG GTGGGTGGGC 60
CAG 63

(2) INFORMATION FOR SEQ ID NO: 10:

20 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
25 (D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CAATGTAGCT GTCGCGACCT GCAAGAAAGA C 31

30 (2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11
(B) TYPE: nucleic acid
35 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GCCAGCAGGC CATGTCACTG G 11

40 (2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 60
45 (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

130

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CGGAATTCTT ACGATGCGTA GTCAGGGACA TCGTATGGGT AGACATCACT TAACATTTTG 60

(2) INFORMATION FOR SEQ ID NO: 13:

5 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: single
10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Ile His Arg Asp Leu Ala Ala Arg Asn
15 5

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 5
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Trp Met Phe Gly Val Thr Leu Trp
5

(2) INFORMATION FOR SEQ ID NO: 15:

30 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4
(B) TYPE: amino acid
(C) STRANDEDNESS: single
35 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Tyr Leu Met Val

40 (2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4
45 (B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

131

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Tyr Val Val Val

(2) INFORMATION FOR SEQ ID NO: 17:

5 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: single
10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Ile His Arg Asp Leu Ala Ala Arg Asn
15 5

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 5
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Trp Met Phe Gly Val Thr Leu Trp
5

(2) INFORMATION FOR SEQ ID NO: 19:

30 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: single
35 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

Tyr Pro Tyr Asp Val Pro Asp Tyr Ala Ser
5 10

40 (2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

45 (A) LENGTH: 11
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

132

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CACAATGTCT TCAAACGCCA C 11

(2) INFORMATION FOR SEQ ID NO: 21:

5 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 63
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
10 (D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GGCTCTAGAT CACGATGCGT AGTCAGGGAC ATCGTATGGG TACTCTGCAG GTGGGTGGGC 60
CAG 63

15 (2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10
20 (B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

25 Tyr Pro Tyr Asp Val Pro Asp Tyr Ala Ser
5 10

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 11
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
35

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GCCAGCAGGC CATGTCACTG G 11

(2) INFORMATION FOR SEQ ID NO: 24:

40 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 60
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
45 (D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

133

CGGAATTCTT ACGATGCGTA GTCAGGGACA TCGTATGGGT AGACATCAGT TAACATTTTG 60

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 31
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
10 (D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CAATGTAGCT GTCGCGACCT GCAAGAAAGA C 31

Claims

1. Isolated, purified, or enriched nucleic acid encoding a nucleic acid encoding PYK2 polypeptide.
2. A nucleic acid probe for the detection of nucleic acid encoding a PYK2 polypeptide in a sample.
3. Recombinant nucleic acid encoding a PYK2 polypeptide and a vector or a promoter effective to initiate transcription in a host cell.
4. Recombinant nucleic acid comprising a transcriptional region functional in a cell, a sequence complimentary to an RNA sequence encoding a PYK2 polypeptide and a transcriptional termination region functional in a cell.
5. An isolated, purified, recombinant, or enriched PYK2 polypeptide having a phosphorylation activity.
6. A purified antibody having specific binding affinity to a PYK2 polypeptide.
7. A hybridoma which produces an antibody having specific binding affinity to a PYK2 polypeptide.
8. A method of detecting a compound capable of binding to a PYK2 polypeptide comprising the steps of incubating said compound with said PYK2 polypeptide and detecting the presence of said compound bound to said PYK2 polypeptide.
9. The method of claim 8 wherein said compound inhibits a phosphorylation activity of said PYK2 polypeptide and is selected from the group consisting of tyrphostins, quinazolines, quinoxolines, and quinolines.

10. A compound capable of inhibiting the phosphorylation activity of a PYK2 polypeptide identified by the method of claim 9.

11. A method of screening potential agents useful for
5 treatment of a disease or condition characterized by an abnormality in a signal transduction pathway, wherein said signal transduction pathway includes an interaction between a PYK2 polypeptide and a natural binding partner, comprising the step of assaying said potential agents for
10 those able to promote or disrupt said interaction as an indication of a useful said agent.

12. A method for diagnosis of a disease or condition characterized by an abnormality in a signal transduction pathway, wherein said signal transduction pathway includes
15 an interaction between a PYK2 polypeptide and a natural binding partner, comprising the step of detecting the level of said interaction as an indication of said disease or condition.

13. A method for treatment of an organism having a
20 disease or condition characterized by an abnormality in a signal transduction pathway, wherein said signal transduction pathway includes an interaction between a PYK2 polypeptide and a natural binding partner comprising the step of promoting or disrupting said interaction.

25 14. The method of claim 13 wherein said disease or condition is selected from the group consisting of epilepsy, schizophrenia, extreme hyperactivity in children, chronic pain and acute pain.

15. The method of claim 13 wherein said disease or
30 condition is selected from the group consisting of stroke, alzheimer's disease, parkinson's disease, neurodegenerative diseases, and migraine.

1/3

FIG. 1.

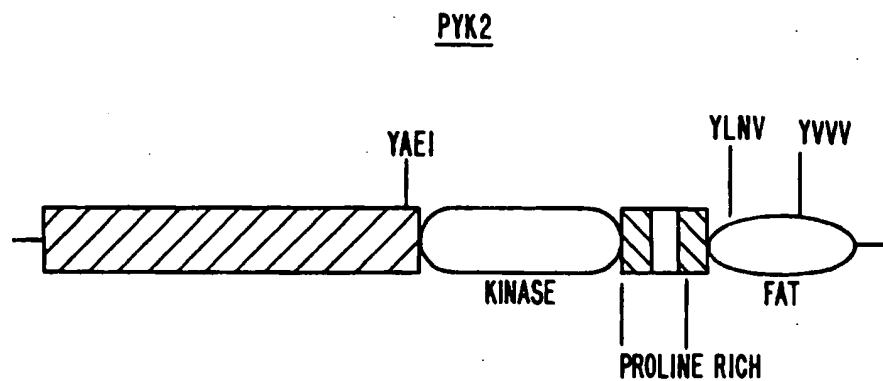


FIG. 2.

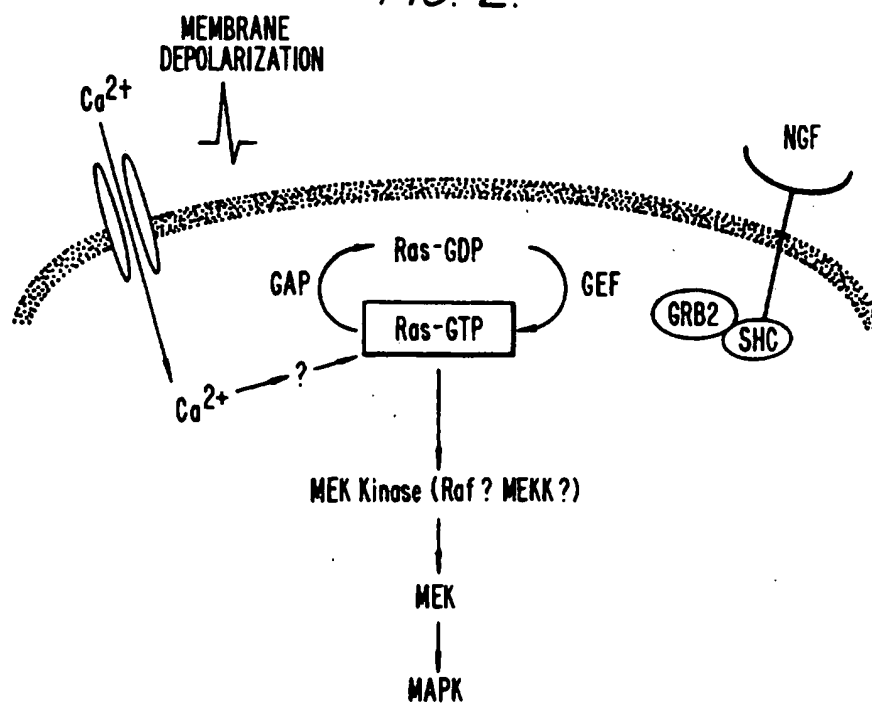
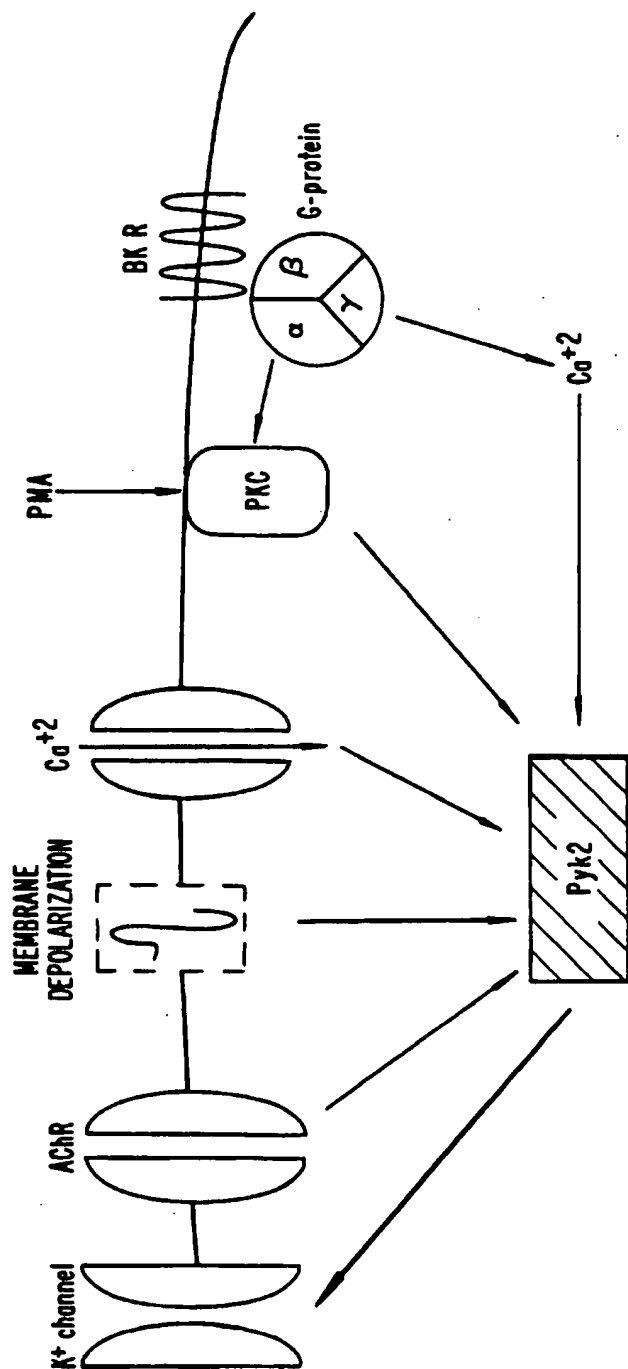


FIG. 3.



3/3

Pyk2	51	V	L	N	R	I	L	G	E	G	E	F	G	E	V	Y	E	G	V	Y	T	N	H	-	K	G	E	K	I	N	V	A	V	K	T	C	K	K	D	G	T	L	D	N	-	K	E	K	F	M	S	E	A	V	
Fak	52	E	L	G	R	C	I	G	E	G	Q	F	G	D	V	H	Q	G	I	Y	M	S	P	-	E	N	P	A	L	A	V	A	I	K	T	C	K	-	N	G	T	S	D	S	V	R	E	K	F	L	Q	E	A	L	
Fer	47	S	L	G	E	L	L	G	K	G	N	F	G	E	V	Y	K	G	T	L	-	-	-	-	-	K	D	-	K	I	P	V	A	V	K	T	C	K	E	D	L	P	Q	E	L	K	I	-	F	L	Q	E	A	K	
HER4	52	K	R	V	K	V	L	G	S	G	A	F	G	T	V	Y	K	G	I	W	V	P	E	G	E	T	V	K	I	P	V	A	I	K	I	L	N	E	T	T	G	P	K	A	N	V	E	-	F	M	D	E	A	L	
Ab1	46	T	M	K	H	K	L	G	G	Q	Y	G	E	V	Y	E	G	V	W	-	-	-	-	-	-	K	K	Y	S	L	T	V	A	V	K	T	L	K	E	D	-	-	-	T	M	E	V	E	E	F	L	K	E	A	A
Pyk2	102	I	M	K	N	L	D	H	P	H	I	V	K	L	I	G	I	I	E	E	E	-	P	T	W	I	I	M	E	L	Y	P	Y	G	E	L	G	H	Y	L	E	R	-	N	K	N	S	L	K	V	L	T	L	V	
Fak	103	T	M	R	Q	F	D	H	P	H	I	V	K	L	I	G	V	I	T	E	N	-	P	V	W	I	I	M	E	L	C	T	L	G	E	L	R	S	F	L	Q	V	-	R	K	Y	S	L	D	L	A	S	L	I	
Fer	99	I	L	K	Q	Y	D	H	P	N	I	V	K	L	I	G	V	C	T	Q	R	Q	P	V	Y	I	I	M	E	L	V	P	G	G	D	F	L	S	F	L	R	K	-	R	K	D	E	L	K	L	K	Q	L	V	
HER4	103	I	M	A	S	M	D	H	P	H	L	V	R	L	L	G	V	C	L	-	S	P	T	I	Q	L	V	T	Q	L	M	P	H	G	C	L	L	E	Y	V	H	E	-	H	K	D	N	I	G	S	Q	L	L	L	
Ab1	99	V	M	K	E	I	K	H	P	N	L	V	Q	L	L	G	V	C	T	R	E	P	P	F	Y	I	I	T	E	F	M	T	Y	G	N	L	L	D	Y	L	R	E	C	N	R	Q	E	V	N	A	V	V	L	L	
Pyk2	155	L	Y	S	L	Q	I	G	K	A	M	A	Y	L	E	S	I	N	C	V	H	R	D	I	A	V	R	N	I	L	V	A	S	P	E	C	V	K	L	G	D	F	G	L	S	R	Y	I	E	D	E	Y	-		
Fak	156	L	Y	A	Y	Q	L	S	T	A	L	A	Y	L	E	S	K	R	F	V	H	R	D	I	A	A	R	N	V	L	V	S	S	N	D	C	V	K	L	G	D	F	G	L	S	R	Y	M	E	D	S	T	Y	-	
Fer	152	R	F	S	L	D	V	A	A	G	M	L	Y	L	E	G	K	N	C	I	H	R	D	I	A	A	R	N	C	L	V	G	E	N	N	T	L	K	I	S	D	F	G	M	S	R	Q	E	D	G	G	V	Y	-	
HER4	156	N	W	C	V	Q	I	A	K	G	M	M	Y	L	E	E	R	R	L	V	H	R	D	L	A	A	R	N	V	L	V	K	S	P	N	H	V	K	I	T	D	F	G	L	A	R	L	L	E	G	D	E	K	E	
Ab1	152	Y	M	A	T	Q	I	S	S	A	M	E	Y	L	E	K	N	F	I	H	R	D	L	A	A	R	N	C	L	V	G	E	N	H	L	V	K	V	A	D	F	G	L	S	R	L	M	T	G	D	T	Y	-		
Pyk2	207	Y	K	A	S	V	T	R	L	P	I	K	W	M	S	P	E	S	I	N	F	R	R	F	T	T	A	S	D	V	W	M	F	A	V	G	M	W	E	I	L	S	F	G	K	O	P	E	F	W	L	E	N	K	
Fak	208	Y	K	A	S	K	G	K	L	P	I	K	W	M	A	P	E	S	I	N	F	R	R	F	T	S	A	S	D	V	W	M	F	G	V	C	M	W	E	I	L	M	H	G	V	K	P	E	Q	G	V	K	N	N	
Fer	204	S	S	S	G	L	K	Q	I	P	I	K	W	T	A	P	E	A	L	N	Y	G	R	Y	S	S	E	S	D	V	W	S	F	G	I	L	L	W	E	T	F	S	L	G	V	C	P	Y	P	G	M	T	N	Q	
HER4	209	Y	N	A	D	G	G	K	M	P	I	K	W	M	A	L	E	C	I	H	Y	R	K	F	T	H	Q	S	D	V	W	S	Y	G	V	T	I	W	E	L	M	T	F	G	G	K	P	Y	D	G	I	P	T	R	
Ab1	204	T	A	H	A	G	A	K	F	P	I	K	W	T	A	P	E	S	L	A	Y	N	K	F	S	I	K	S	D	V	W	A	F	G	V	L	L	W	E	I	A	T	Y	G	M	S	P	Y	P	G	I	D	R	S	
Pyk2	254	D	V	I	G	V	L	E	K	G	D	R	L	P	K	P	D	L	G	P	P	V	L	Y	T	L	M	T	R	C	W	D	Y	D	P	S	D	R	R	R	R	F	T	E	L	V	C	S	L						
Fak	255	D	V	I	G	R	I	E	N	G	E	R	L	P	M	P	P	N	C	P	P	T	L	Y	S	L	M	T	K	C	W	A	Y	D	P	S	R	R	P	R	F	T	E	L	K	A	Q	L							
Fer	251	Q	A	R	E	Q	V	E	R	G	Y	R	M	S	A	P	Q	N	C	P	E	E	I	F	T	I	M	M	K	C	W	D	Y	K	P	E	N	R	P	K	F	S	D	L	H	K	E	L							
HER4	256	E	I	P	D	L	L	E	K	G	E	R	L	P	Q	P	P	I	C	T	I	D	V	Y	M	V	M	V	K	C	W	M	I	D	A	D	S	R	P	K	F	K	E	L	A	A	E	F							
Ab1	251	Q	V	Y	E	L	L	E	K	D	Y	R	M	K	R	P	E	G	C	P	E	K	V	Y	E	L	M	R	A	C	W	Q	N	P	S	D	R	P	S	F	A	E	I	H	Q	A	F								

FIG. 4.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☒ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.